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Characterization Ofp53 Gene Sequence in Exons 5-8 of the Western Mosquito Fish, Gambusia Affinis.

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CHARACTERIZATION OF p53 GENE SEQUENCE IN EXONS 5-8 OF THE WESTERN MOSQUITO FISH, *GAMBUSIA AFFINIS*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment for the degree of
Doctor of Philosophy

in

The Inter Departmental Program in Veterinary Medical Sciences through the
Department of Veterinary Physiology, Pharmacology & Toxicology

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Dedicated to
My Family

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Abstract

Cancer is a multistage disease that involves both genetic and epigenetic factors. The number of physical and chemical agents with which human beings come in contact on a regular basis is increasing everyday. Somatic mutations can result due to such exposures and trigger uncontrolled cell division resulting in cancer.

The p53 tumor suppressor gene has been identified in several vertebrate species ranging from man to fish. In addition, inactivation of p53 has been observed in a wide variety of tumors. Fish models are becoming increasingly popular for assessing environmental exposure. Low background incidence of mutations, relatively low cost tumor studies, and the ability to extrapolate the results to humans makes these models viable alternatives. The Western mosquito fish (*Gambusia affinis*) a fresh water species (order Atheriniformes; family Poeciliidae) was chosen as a model organism for this study.

Polymerase chain reaction with rainbow trout genomic DNA as a positive control was conducted. Fragments encompassing exons 5-6 and 7-8 were isolated and sequenced. Alignment of the sequences (exons 5-8) of the mosquito fish with that of rainbow trout in the similar regions revealed high homology between the species.

Southern transfer of restricted genomic DNA of *Gambusia affinis* was conducted (target DNA). A PCR product (exons 5-6) of 450 base pairs was digoxigenin (DIG) labeled (probe DNA). In a separate set of experiments, PCR product from *Gambusia affinis* exons 7-8 (target DNA) was probed with another DIG-labeled PCR

product (350 base pairs) from similar regions of rainbow trout genomic DNA. Hybridization of the probe and target DNA followed by chemiluminiscent detection resulted in visualization as bands on X-ray film indicating high homology between mosquito fish and rainbow trout p53 gene.

While rainbow trout can function only in cold-temperatures, medaka, being exotic, is restricted to use only in the laboratory. In the present work the mosquito fish can withstand temperature ranging from 40 ° F - 110 ° F. In addition, this study can help promote the concept of the mosquito fish as a sentinel species for environmental monitoring and replace rainbow trout and medaka for direct validation in field.

Chapter 1

Introduction

Despite considerable progress in understanding the genetic mechanisms of cancer, the applications in combating life-threatening situations are still inadequate. Based on epidemiological evidence environmental factors account for nearly 75,000 modern day cancers (Doll and Peto, 1981). According to a report on cancer research and its impacts (Reines, 1986) it is estimated that on average, 50,000 man-made chemicals are in use with nearly 500-1000 new ones being introduced into the market each year. Oil and gas production, power plants and associated wastes (produced waters) all contribute to environmental contamination on a large scale. Crude oil and coal contain a large number of polycyclic aromatic hydrocarbons (PAHs) at low concentrations. However, large amounts of these compounds are produced by pyrolysis and combustion processes. Many of the PAHs are recognized as potent carcinogens (Harvey, 1982). These chemicals to varying degrees enter the water, air, soil and food supply and contribute to the constant assault on the ecosystem. The aquatic sediments due to surface runoff from land, atmospheric fall out or emissions from diesel engines or pumps invariably contain PAHs in varying concentrations. However, at sites associated with spills, refinery effluents, oil and gas production, and sediment PAH concentration are an order of magnitude higher. This kind of exposure of xenobiotics to the living organisms can be at sublethal or lethal doses over a period of time resulting in cellular alterations at the molecular level (DNA or RNA) that can be both

reversible and/or irreversible. In the latter situations, cancer can result. Cancer is a complex multistage process that involves both genetic and epigenetic events. Genetic events include disruption of the cellular genes which on activation (protooncogenes) and/ or inactivation (tumor suppressor genes) can trigger a series of events that finally culminate in a tumor. In the initial stages of tumor production, susceptible cells gain a selective growth advantage over neighboring normal cells and undergo clonal expansion. In certain situations genomic instability occurs as the clones turn malignant and metastasize to different parts of the genome (Harris, 1991 and 1996).

The increasing awareness of the potential adverse effects upon the large-scale use of chemicals by many agencies has resulted in the Clean Air Act Amendments of 1990. The National Toxicology Program (NTP) was mandated by Congress to perform certain tests in a limited period of time (5 years) to assess the toxic potential of a large number of chemicals. Many administrators realized that it would be almost impossible to achieve this mandate in terms of both time and money with the currently available procedures. Most of the traditional animal toxicity testing is generally done with the use of mammalian models that have relatively long life spans (1-3 yr.). Hence, these studies are also very expensive and time consuming. Short-term *in vitro* tests such as Ames tests (Ames et al., 1973, 1990 and Ames, 1983) have the advantage of being economical and rapid. However, their use becomes limited when it becomes necessary to evaluate such variables as bioaccumulation, tissue distribution, and metabolism and to determine target organ specificity. Also these tests often yield false negative and false positive results that limits their validity (Goss and Sabourin 1985).

As a response to some of these problems, fish have been targeted as alternate models for research. They have the advantage of having shorter life spans, and are also less expensive to use in toxicity testing. Many reports have indicated fish to develop liver neoplasia on exposure to environmental contaminants such as hepatocarcinogens. It is the opinion of many investigators that in many cases of environmental contamination, a positive correlation exists between water pollution and incidence of tumor formation (Harshbarger and Clark, 1990; Harshbarger et al., 1993). Neoplasms have been documented in nearly all the major organs of the fish and have also been found to be similar to humans histologically (Van Beneden et al., 1990). However, although in mammalian species a relationship between exposure to carcinogens and alterations in cellular oncogenes has been established, few such studies exist for aquatic species. Among fish, small sized fish are increasingly becoming popular as bioassay models for carcinogenicity testing. In addition to having the same advantages as the other members of their phyla, small sized fish need only a relatively short time to develop tumors (1-2 yr.) and also possess a very low spontaneous tumor rate. Many small fish have been used in toxicology testing which include medaka (*Oryzias latipes*), guppy (*Poecilia reticulata*), sheepshead minnow (*Cyprinidon variegatus*) and *Gambusia affinis* (Schultz and Schultz, 1982 and Law et al., 1994).

The molecular study of carcinogenesis may be pursued either by focusing on the oncogenes or by studying tumor suppressor genes. Both the genes are involved in the regulation of normal cellular growth. Mutagenic events occurring in these genes may result in activation of oncogenes and/or inactivation in the case of tumor suppressor genes that can ultimately result in tumorigenesis.

Among the tumor suppressor genes that have been characterized in mammals a few include Rb (retinoblastoma gene), p53, APC (adenomatous polyposis coli), NF1 (neurofibromatosis type 1 gene), WT (Wilms's Tumor gene), and the DCC (deleted-in colon carcinoma gene). The p53 gene was chosen as the focus of this study due to its important role in controlling the cell cycle and also because it has been found to be mutated in more than 51 types of human tumors. A few tissues where the gene has found to be mutated in human tumors include breast, colon, esophagus, liver, bladder, ovary, prostate, skin, stomach, thyroid and brain. The p53 gene sequence although has been primarily reported in vertebrates, the presence of putative genes have been reported both in squid (*Loligo forbesi*) and clam (*Mya arenaria*) (Soussi and May, 1996). Five conserved regions (domains) that also correspond to the mutational hotspots have been reported in all species in which the p53 gene was studied.

A goal of the research in our laboratory has been to identify a native fresh and salt-water species that could be used as a bioassay model for mutagenesis and carcinogenesis studies. Mosquito fish, (*Gambusia affinis*) is native to the U. S. and has a wide temperature tolerance. In addition to being small in size, it has been reported to be resistant to a wide variety of diseases and has been found to develop tumors rapidly. Law et al., have studied the mosquito fish (*Gambusia affinis*) as a potential model for carcinogenicity testing. In their studies, p53 expression was found to increase in fish exposed to the promutagen methylazoxymethanol acetate (MAM-AC) as opposed to the control (unexposed) fish.

1.1 Objectives

Exposure of organisms including man to environmental contaminants can cause damage to DNA that can be used as a biomarker to evaluate genotoxic properties of the chemical. Most traditional carcinogenicity testing has been conducted using mammalian models. Fish species have only been recently recognized as alternatives for some situations. There is a need to select sentinel fish species that have wide spread geographic distribution, that are easy to maintain and which exhibit responses or measurable biomarkers that can be studied upon exposure to contaminants in a variety of environments including freshwater, estuarine and marine environments. Small fish species are viewed as a way to reduce the large number of animals needed for traditional carcinogenicity testing using mammalian models and as a tool to address issues of the effects on low-dose chronic exposures to carcinogens.

In previous studies in our laboratory, Law et al., have been successful in demonstrating *Gambusia affinis*, the Western mosquito fish, as a possible model organism for carcinogenicty testing. They reported the ability of tumor induction in as short as 26 weeks of exposure to MAM-Ac (Law et al., 1994). In further studies, they were able to demonstrate a relationship between DNA adduct frequencies and tumor prevalence (Law et al., 1997). Also, these fish are widely distributed and also easily cultured in the laboratory. It is increasingly being recognized that the molecular events that occur in an organism during the early periods of exposure to chemicals are important. In addition to studies involving immunochemistry, Law et al., (1994) observed that it is also important to study inactivation of p53, the tumor suppressor gene and that this response in fish could be used as a potential biomarker of exposure to

environmental contamination. The p53 gene has been widely studied in mammalian species, and has been used as a bioindicator, where the gene has been well characterized. Among fish, however, p53 gene has been studied only in rainbow trout and medaka. The primary objective of our research was to develop *Gambusia affinis*, as a sentinel fresh water species for carcinogenicity testing. The specific objectives of these studies included:

1) Identifying and sequencing genomic sequence of the p53 gene in *Gambusia affinis* in exons 5-8 (conserved regions).

2) To conduct Southern hybridization on genomic DNA of *Gambusia affinis* to confirm the presence of the p53 gene.

3) To screen the p53 gene sequence for mutational events using tumors from *Gambusia affinis* exposed to MAM-Ac.

Chapter 2

Literature Review

The study of the various intricate mechanisms involved in the development of cancer is being undertaken with a holistic approach. In this process, major emphasis is placed on theories of mutation, selection, oncogenes, tumor suppressor genes and aberrant differentiation. All these theories have been studied and reported in numerous papers (Vogelstein and Kinzler, 1992; Harris 1991). A few of the milestones will be reviewed with special emphasis on hepatocarcinogenesis to provide a basis for understanding the mechanisms of cancer being investigated in the present study.

2.1 Carcinogenesis: A Perspective

Carcinogenesis is defined as a complex multistage process wherein normal growth, differentiation and development have gone awry (Harris, 1991). It basically involves a minimum of 3-7 events (genetic and epigenetic) which provide selective growth advantage to the susceptible cells to undergo clonal expansion (Vogelstein and Kinzler, 1992; Harris, 1996).

Peraino and colleagues first documented the sequential development of the stages of liver cancer in 1971. They reported the use of phenobarbital as a promoter in hepatocarcinogenesis in rats. In other studies, Solt and Faber in 1976 developed a resistant hepatocyte model to explain the stages of hepatocarcinogenesis. This method can be explained on the basis that the liver is made up of hepatocytes that differ in the

bio-chemical functions. As such, a subpopulation of cells with a particular biochemical function will react differently to the toxic effects of a given chemical (e.g. 2-acetylaminofluorene, 2-AAF) as opposed to the rest of hepatocytes. Faber and colleagues in 1971 explained that certain cells in rat liver are resistant to cytotoxicity either due to reduced xenobiotic activation or enhanced detoxification mechanisms. Also, the ability of a small percentage of cells to acquire selective growth advantage over the neighboring normal cells (neoplastic properties) results in clonal expansion.

2.1.1 Stages of Carcinogenesis

The process of carcinogenesis has been studied extensively in animal models and consists primarily of 3 stages: tumor initiation, promotion, and progression (Harris, 1991).

- Tumor Initiation: As a result of exposure to carcinogens (chemical, physical or microbial) normal cells undergo genetic changes to form initiated cells. These initiated cells (clones) acquire selective growth advantage in expansion as opposed to the surrounding neighboring normal cells, and thus, disrupt the normal architecture of the cells and interfere with the signal transduction pathways necessary for homeostatic growth (Yuspa et al., 1986; Harris.1991). This stage intrinsically explains the interaction of DNA of the target cell and the carcinogen (genotoxic carcinogen).
- Tumor Promotion: The second stage of carcinogenesis involves extensive proliferation of the initiated cells. During the process of replication, the population of initiated cells that harbor the endogenous mutations increase resulting in additional genetic damage. Subsequent exposures to DNA-damaging events can increase the

odds of the initiated cells to turn malignant. This process can occur by activation of the protoncogenes and or inactivation of tumor suppressor genes. The presence of the malignant cells can create genomic instability that is manifested as aneuploidy (abnormal number and structure of chromosomes, gene amplification, and altered gene expression (Harris, 1991).

- Tumor Progression: In the latter stages, angiogenesis occurs and the tumor grows to 1-2 mm in size. Metastasis then results that helps in disseminating cells through vessels to distant tissues (Figure 2.1, The multistage process of carcinogenesis).

2.2 The p53 Tumor Suppressor Gene

The role of protooncogenes and tumor suppressor genes in cellular processes (cell growth, cell cycle and cell differentiation) is becoming increasingly apparent. The proper functioning of the cell is dependent on the genes being present in the appropriate form. Both protooncogenes and tumor suppressor genes are cellular genes under normal circumstances. However, inappropriate activation in the former and inactivation in the latter can result in neoplastic transformation. While activation of protooncogenes results in base substitution mutations, chromosomal translocations, and gene amplifications, in the case of recessive tumor suppressor genes, inactivation of the two alleles is caused either by base substitution mutations, deletions, chromosomal nondysjunction and or recombinations (Aaronson, 1991; Harris, 1991 and Weinberg, 1991). This indicates that the tumor suppressor genes undergo more mutational events when compared to protooncogenes and hence the odds of them being targets for certain carcinogens is greater.

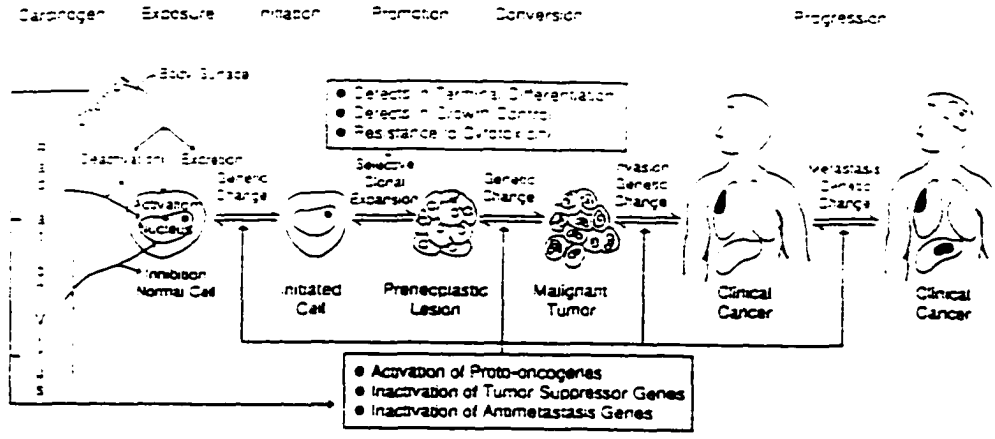


Figure 2.1 Multistage process of carcinogenesis
(adapted from Shields and Harris, 1991)

2.2.1 Brief History

The tumor suppressor gene, p53 is a nuclear phosphoprotein and was originally discovered in Simian virus 40 (SV40) transformed cells (Linzer and Levine, 1979). The p53 protein is 53 Kda in molecular weight (Hollstein et al., 1991) and due to its ability to bind strongly with large T antigen, was identified first as a tumor antigen. In subsequent studies, it was reported that p53 could function as an oncogene due to its ability to transform primary rat embryo fibroblasts in cell culture. More recent studies proved that both the roles of p53 identified were inaccurate due to the fact that the clones the investigators were studying contained mutant copies of the gene. It is now agreed among researchers that the p53 gene in its wild-type form is a tumor suppressor gene and regulates the cell growth and division in a negative fashion. However, in addition, unlike other tumor suppressor genes such as the retinoblastoma gene, alterations in p53 can also result in a function of the protein to enhance (promote) cell division (Hollstein et al., 1991).

Cells can be transformed by interaction with cellular and viral proteins such as SV40 large T antigen, the adenovirus 5E1b protein, the E6 protein of the human papilloma virus 16 and 18 and the 70 KD family of the heat shock proteins (Linzer and Levine, 1979; Lane and Crawford, 1979). In addition, cells with mutant p53 can also be transformed on exposure to chemical carcinogens. These transformed cells often contain elevated levels of p53 protein and this altered protein has found to be more stable in comparison to the wild-type p53 found in non transformed cells. This property of altered stability can thus be exploited as a biomarker in tumor studies.

The wild-type p53 protein has a relatively shorter half-life of about 40 minutes in normal cells (Deppert and Haug, 1986) and is also relatively unstable when compared to the more stable mutant p53 which has a half-life of a few hours (Takahashi et al., 1991). The p53 protein has a tendency to self-aggregate and hence exists as a tetramer. It also forms complexes with agents such as DNA tumor viruses, RNA viruses and chemical carcinogens that enhance its stability and prevent it from degradation. Although transformed cells have been positively correlated with increased p53 stability, these increases are independent of any particular mutation of the p53 gene (Halevy et al., 1989).

2.2.2 Functions of the p53 Protein

The functions of p53 are still being elucidated. One of the most interesting functions is that it appears to participate in the initiation of programmed cell death (apoptosis) in response to DNA damage. The p53 gene has also been reported to be involved in cell cycle control, DNA repair and synthesis and cell differentiation (Harris and Hollstein, 1993). While the presence of the wild-type allele has been found to suppress carcinogenesis, its inactivation can result in uncontrolled cell division that results in tumor formation. Several investigators have reported that wild-type p53 functions to protect the genome against DNA damage by sensing the damage and halting the cell division until the damage is corrected. In situations where damage is extensive, p53 can trigger apoptosis. These events serve to maintain the integrity of the cell and to prevent mutated DNA from being propagated on to daughter cells. However, if the p53 gene itself gets mutated, the genome guarding property may be

inactivated and the cell continues to accumulate DNA damage. In such situations, p53 can function as an oncogene that can be doubly dangerous to the cell.

2.2.3 The p53 Gene and Cell Proliferation

Research on p53 protein is a primary focus of research in molecular biology. It is universally accepted that the inactivation of the tumor suppression activity of p53 is associated with the progression of cancers. In an effort to elucidate the detailed role of p53 in cell regulation, p53 has been identified as a transcription factor that binds to other gene promoters/promotion complexes and regulate their expression (Culotta and Koshland, 1993). Momand et al., (1992), reported that a rat protein MDM2 coprecipitated with p53. The MDM2 gene and promoter region was later mapped to the long arm of human chromosome 12 and was also found to bind to p53 protein. MDM2 was initially classified as a dominant transforming oncogene and was found to act in a negative feedback loop involving p53. In gene amplification studies, Barak and Oren et al., (1992) reported that in those tumors where MDM2 was amplified, no mutations in p53 were found. In other words, wild-type p53 protein can keep a check on MDM2 expression until needed for cell replication. However, any dysregulation of the feed back loop results in MDM2 being overexpressed resulting in cell proliferation and potentially in cancer. Of the soft-tissue sarcomas assayed, MDM2 was expressed in 30 percent.

2.2.4 The p53 Gene and its Implications in the Cell Cycle

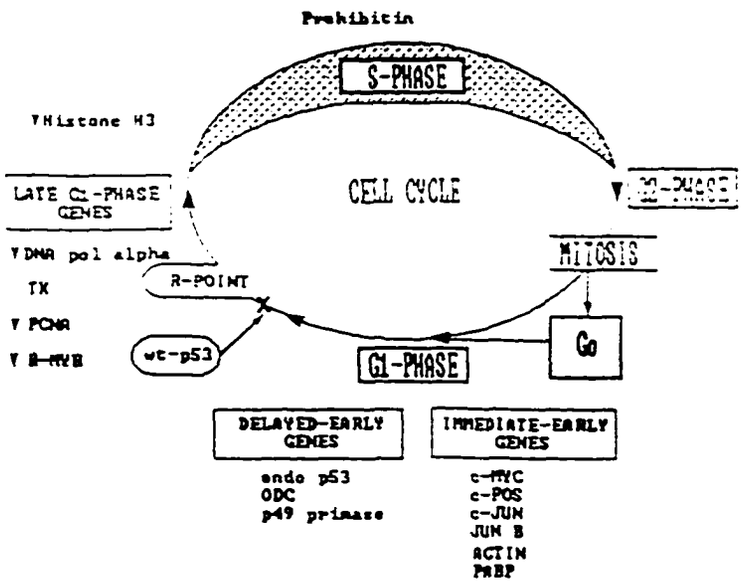
The important role of p53 in carcinogenesis and as a potential biomarker in risk assessment substantiates the need to know about its detailed involvement in the cell cycle. An important function of p53 is its involvement in regulating the cell cycle with

wild-type (wt) p53 serving as a "check point" in regulating cell division. Two major checkpoints were reported in mammalian cells that regulate cell proliferation. The first check point, was found to occur early in the cell cycle when the cells exit the resting cycle and enter G_0. The second check point, also called the restriction point, occurs later in the G_1, but before the cells enter the synthesis phase (S-phase). This step is necessary to provide additional time for the cell to repair any damage that may have occurred to DNA (Mercer et al., 1991). This can also result in the selective down regulation of the expression of the proliferating cell nuclear antigen (PCNA), which is a component of the DNA replication system (Mercer et al., 1991). A schematic representation of involvement of p53 in the cell cycle is depicted in Figure 2.2

The p53 gene has also been studied for its role involving other components of the cell cycle. The proteins called cyclins and the associated enzymes, the cyclin dependent kinases were found to associate with p53. In these studies, two teams of scientists working independently identified a new gene. The gene was named WAF1/Cip1 and p53 was found to promote its expression. The protein product of the WAF1 gene (p21) was found to bind to cyclin dependent kinases and inhibit their action. This process can also halt the cell cycle and thus provide the cell with some additional time for repair of DNA damage.

2.2.4.1 Subcellular Distribution of the p53 Protein

The subcellular localization of p53 is dependent upon the stage of the cell during mitosis. In cells that are undergoing mitosis, p53 is found localized in cytoplasm during the G_1 phase but during the early stages of S-phase is localized in the nucleus. As the S phase progresses, p53 is transported back to the cytoplasm



Model for control of cell cycle progression by wt-p53 protein. Shown is a schematic diagram depicting the effect of wt-p53 protein on the expression of growth-response genes. The point in the cell cycle during the Go to S-phase transition where the genes are maximally expressed is indicated by their position in the diagram. The genes affected by wt-p53 protein expression are shown with arrows illustrating down-modulation of their mRNA levels. The approximate position of the restriction point (R-point) in late G1-phase is shown.

Figure 2.2 Schematic representation of cell cycle (adapted from Mercer et al., 1991)

(Shaulsky et al., 1990). These results were substantiated in studies utilizing temperature-sensitive mutant cell lines that behave either as the wild-type form or the mutant form depending upon the temperature of the experiment. At 32 ° C (sub-optimal) growth arrest occurs and p53 was found in the nucleus, whereas, at 37 ° C (optimal for S-phase), proliferation of cells occurred and p53 was found primarily in the cytoplasmic fraction (Gannon and Lane, 1991).

2.2.5 Properties of p53 Protein

2.2.5.1 Biochemical Properties

In response to damage, cells may modulate the functions of proteins by post-translation modifications that include phosphorylation and dephosphorylation (Cox and Lane, 1995). The p53 protein is a nuclear phosphoprotein and in SV40 transformed cells was reported to have two-fold higher phosphorylation than from non-transformed cells. Reports on p53 protein from mouse cells transformed with SV40 antigen reveal phosphorylation at multiple sites on the polypepetide indicating a possible role of the phosphorylation in cell transformation. Phosphorylation of the p53 protein can also regulate the cell cycle. Under phosphorylation of p53 is higher in G_0/G_1 stage (s), when compared to the S phase. Phosphorylation of p53 might result in the transition of the cells from G_0/G_1 to the S phase (Bischoff et al., 1990).

2.2.5.2 Immunological Properties

Although p53 is a cellular-encoded protein, in certain situations it is reported to be immunogenic with specific antibody production. Among the antibodies raised to p53 from mammalian cells, the wild-type p53 is recognized by PAB246, PAB607 or PAB1620, while the mutant form is recognized by PAb240 (Montenarh, 1992).

Denaturation of the p53 protein resulted in the destruction of the epitopes and absence of recognition by the antibodies specific for the wild-type conformation. These studies were corroborated when a change in temperature caused a temperature-sensitive mutant of p53 to change its conformation from Pab246^{+} to Pab246^{-} (Millner and Medcalf, 1991). Some of these antibodies are species specific and others have unique antigenic determinants and show cross-reactivity between species. The alternative structural conformations of the p53 polypeptide in response to mutation induced changes have to considered in recognizing the various epitopes of the antibodies.

2.2.6 Functional Aspects of p53 Protein DNA-Binding Properties

While wild-type p53 was found to bind to both double stranded and single stranded DNA, the mutant p53 proteins were found to bind more to single stranded DNA. These reports indicate that some specific conformation is necessary for the p53 protein to bind to DNA (Steinmeyer and Deppert, 1988).

In vitro experiments with human DNA resulted in identification of two protein sequences that are essential for the specific DNA binding. The first is associated with a 33 base repeat and the second consists of two repeats of the bases TGCCT (Kern et al., 1991a). In the 33 base pair sequence, guanine containing codons associated with certain amino acids were found to be more critical for DNA binding than the other bases.

2.2.7 Mutations in the p53 Gene

2.2.7.1 Germ-Line Mutations in the p53 Gene

Although mutations in p53 are commonly somatic in origin, for some cancers such as Li-Fraumeni, the p53 mutations appear to be heritable. Li-Fraumeni syndrome

is a genetic disorder, where the affected individuals inherit one wild-type p53 gene and one mutated p53 gene (Donehower et al., 1992). This condition is epidemiologically characterized as being rare and until now only six families have been identified as having this disorder. While the germ cells of the affected individuals are heterozygous for wild-type and mutant p53, the tumors resulting from the disease process are homozygous for mutant p53. Although the heterozygous condition does not interfere in general with the normal functions of the affected individuals, they are prone to secondary mutations in the cells that harbor the first mutation. If a mutation occurs in both alleles, then the individuals develop neoplasms in different parts of the body. An analysis of the mutation spectrum in these individuals revealed that most of the mutations are clustered between codons 245 and 248 of the p53 gene (Malkin et al., 1981).

Experiments to substantiate the findings in humans were conducted using a mouse model system in which the normal endogenous p53 alleles were deleted using gene knock-out technology (Donehower et al., 1992). Similar to the conditions of individuals with the Li-Fraumeni syndrome, the mice that were homozygous for the p53 deletion appeared normal but were prone to a variety of tumors as early as six months of age (Baker et al., 1990). The mice that were heterozygous containing one wild-type and one mutant allele, were also prone to develop tumors but at a reduced frequency and a lower rate when compared to mice with the homozygous mutant allele (Srivastava et al., 1990).

2.2.7.2 Somatic Mutations

Mutations occurring in the p53 gene are largely (>98%) missense mutations, where a single base pair substitution occurs. Other types of alterations such as loss of

alleles, rearrangements and deletions have also been reported in the p53 gene in a few cases. Base pair substitutions can be of two types. The first type is a transition where a purine is replaced by purine or a pyrimidine is replaced by a pyrimidine, and the second type is a transversion where a purine replaced by pyrimidine or *vice versa*. Base pair substitutions can result in the alteration of a codon that may lead to a change of amino acid of a protein. In certain situations such as with oncogenes or tumor suppressor genes this ultimately results in tumor formation. The importance of p53 as a biomarker substantiates the need to study its mutational spectrum. Several investigators have confirmed that both endogenous and exogenous events cause base pair substitutions in the p53 gene. The frequency and type of p53 mutations can thus, act as a molecular dosimeter and provide insight into the possible origins of the mutations that finally culminate in a tumor (Hollstein et al., 1994).

2.2.7.3 Endogenous Events

Some of the endogenous events which may finally result in the generation of point mutations in the human cell include DNA polymerase infidelity (Loeb and Cheng, 1990), depurination (Loeb and Preston, 1986), oxidative damage due to free radical production (Lutz, 1990, Breimer, 1990; Hollstein et al., 1991) and deamination of 5-methyl-cytosine (Ehrlich et al, 1990). In many cases of colon cancer, a high frequency of C to T transitions were observed due to the presence of 5-methyl cytosine residues in the CpG dinucleotide sequences. In the cell, oxidative damage results from the production of active oxygen species mediated through hydrogen peroxide, superoxide anions (O_2^-) and hydroxy radicals (OH^-) commonly found in cells associated with inflammation (Hollstein et al., 1991).

2.2.7.4 Exogenous Events

The exogenous events category which may cause mutations include exposure to carcinogens which in turn cause 'mutation finger print' in DNA (Hollstein et al., 1994) where only specific codons are effected. These types of base substitutions have been documented in bacteria, mammalian cells *in vitro* and experimental animals (Hollstein et al., 1994). Many environmental carcinogens are electrophiles or are converted to electrophilic metabolites and can attack nucleophilic DNA bases to form so-called DNA adducts. During the process of cell replication this damage can be fixed at the site of the adducted base in the form of an alteration of the base sequence. Once a mutation is fixed the misincorporated base sequence would be passed on to the daughter cells of the damaged cell. Several classes of environmental carcinogens have been studied for their ability to mutate and thus inactivate p53. Polycyclic aromatic hydrocarbons (PAHs) because of their widespread occurrence are among the chemicals that have been selected to study p53 inactivation. A few examples include aflatoxin B1 (Cho et al., 1994) and polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene and dimethyl benzantharacene (Ruggeri et al., 1991, and 1994), N-nitrosodiethylamine and 4-aminobenzene (Goodrow et al., 1992).

2.2.8 Evolutionary View of the p53 Gene

The importance of p53 can also be understood by studying its evolutionary conservation among phyla. Until recently, p53 gene been reported to be conserved only in the vertebrate species ranging from man to fish (Soussi et al., 1990). However, recent studies have aimed at trying to isolate the gene in invertebrates. A putative p53 gene in a clam (*Mya arenaria*) (P. Winge and S. Friend, personal communications in

Soussi and May, 1996) and squid (*Loligo forbesi*) (Ishioka et al., 1995) have been identified. Also using degenerate oligonucleotides and low stringency PCR amplification, p53 has been cloned in certain mollusks (P. Winge and S. Friend personal communications in Soussi and May, 1996). These discoveries indicate other lower phyla of the animal kingdom to possess the p53 gene than previously thought and that it should only be a matter of time and technique to identify the same in other species. However, p53 genes or a close functional analog have not been reported in sea urchin or *Drosophila melanogaster* (Soussi et al., 1990, 1989). Reports of studying the p53 gene in yeast have indicated that although p53 was not detectable in yeast, human wild-type p53 was found to inhibit cell division in *Saccharomyces cerevisiae* and *Saccharomyces pombe*, whereas the mutant p53 did not induce a detectable phenotype (Wagner et al., 1991; Bischoff et al., 1992). The ability to activate transcription is an important property of p53 protein. Thiagalingam and his colleagues (1995) used genetic selection to isolate a mutant in *Saccharomyces cerevisiae* that was defective in p53 mediated transcriptional activation. The defect was partially corrected with a yeast gene named PAK1 (p53 activating kinase). The PAK1 gene has similar regulating functions as that of the genes involved in cell cycle control. The discovery of PAK1 in yeast indicates that other types of genes that regulate p53 activity *in vivo* may be present. The ability to detect p53 in some invertebrates and not others raises interesting questions about the role of p53 in an organism's life cycle and the techniques performed for the successful identification of the gene in some organisms.

2.2.9 Molecular Organization of the p53 Gene

Among vertebrate's the p53 gene has been isolated and sequenced in twenty three species up to date (Soussi and May, 1996). In five species, p53 gene has been cloned completely (Matlashewski et al., 1984; Bienz et al., 1984; Lamb and Crawford, 1986; Soussi et al., 1990; Hulla and Schneider, 1993 and Albor et al., 1994). Five conserved regions in the p53 gene in vertebrates have been identified and correspond to exons 5-8 (Soussi et al., 1990; Soussi and May, 1996). These regions correspond to domains I to V of the protein and include 23 amino acids (domain IV) and 17 amino acids (domain V). The intervening sequences (introns) were not conserved. While domains I, III, IV and V were found to be correspond to exons 2,5,7 and 8 of the p53 gene respectively, domain II corresponds to exon 4 and 5 (Figure 2.3). Interestingly, among the conserved regions, certain sites of amino acids were found to be targets for mutations and were labeled as "hot spots". The regions associated with amino acid sequences are also sites of protein: DNA interactions and play an important role in stabilizing the protein (Soussi et al., 1990; Greenblatt et al., 1994).

A striking similarity was observed among different species in terms of genomic organization of the p53 gene. In human, mouse and *Xenopus laevis*, the presence of eleven exons interrupted by ten introns at precise homologous positions was reported (Soussi et al., 1990). Hulla and Schneider (1993) reported the rat p53 to have only ten exons and nine introns. The same investigators reported an allelic polymorphism within intron 5. The intervening sequence (intron) in between exons 5 and 6 was missing in the rat gene when compared to human and mouse p53 genes. Hulla and Schneider speculate that intron 6 may be of less evolutionary significance because it is

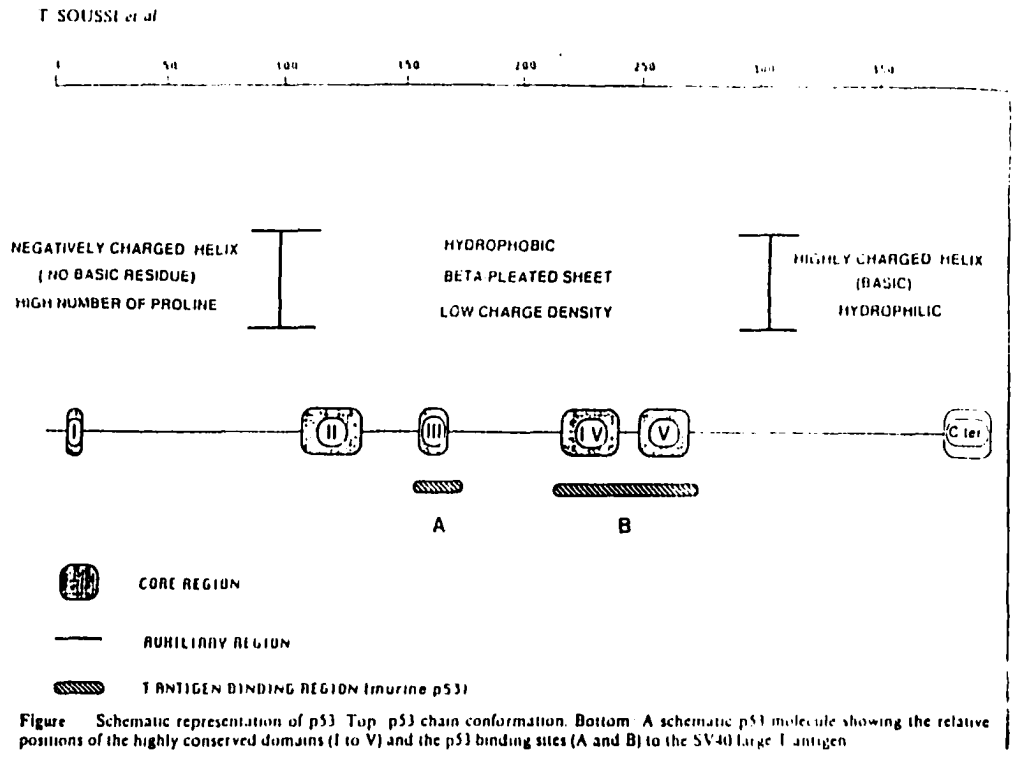


Figure Schematic representation of p53. Top: p53 chain conformation. Bottom: A schematic p53 molecule showing the relative positions of the highly conserved domains (I to V) and the p53 binding sites (A and B) to the SV40 large T antigen.

Figure 2.3 Schematic representation of p53 gene (adapted from Soussi et al. 1992)

not conserved in all the species. However, they reported the gene structure to be conserved to a large extent. The species characterized so far differed in the degree of homology in the p53 gene. There was a higher homology in the p53 gene between human and mouse (>80%) when compared to human and fish (47%) (Caron de Fromentel (1992).

Another interesting feature of the p53 gene that has been reported to be universal both in normal as well as transformed cells, is the ability to exist both as regularly and alternatively spliced sequences (Han and Martin, 1992). In studies in mouse liver and testis, alternatively spliced mRNA sequences transcribed from p53 gene were found to contain an additional 96 bases derived from intron 10 and to be present at 25 to 30% of the level of mRNA transcribed with regularly spliced p53 gene sequence in normal epidermal and carcinoma cells.

Diploid organisms, such as humans and mouse, have one active gene whereas, the amphibian *Xenopus laevis*, being a tetraploid organism, has two active gene copies. In man, the p53 gene is found on chromosome 17 at position 17p13.1 (Benchimol et al., 1982; Isobe et al., 1986), while the functional gene in mouse occurs on chromosome 11 with an inactive pseudogene on chromosome 14 (Zakut-Houri et al., 1983). In case of the rat genome, at least two p53 pseudogenes have been identified in addition to the functional gene (Hulla 1992, Schneider et al., 1991 and Hulla and Schneider, 1993). According to Lewin (1990), the pseudogenes can be defined to arise through a mechanism whereby a spliced mRNA is reverse transcribed and subsequently inserted into the genome. For this reason, these pseudogenes may contain inactive, noncoding exons and 3' untranslated regions but do not contain introns. In

recent studies on rat p53 gene, conducted by two teams of scientists in Singapore and the United States, the cloning and characterization of p53 pseudogenes from the rat genome have been reported. The former group of investigators has been successful in cloning *via* the Polymerase chain reaction (PCR), two rat genomic fragments of 1.3 and 1.2 kb respectively. These two pseudogenes designated as ΨR53-1 and ΨR53-2 respectively were found to lack intron sequences and represented a start to stop codon length of the processed transcript of the functional p53 gene. In hybridization experiments, the pseudogene sequences were found to hybridize with the human cDNA. Nucleotide sequencing revealed that the two genomic DNA pseudogenes share 85% and 83% homology respectively with the rat cDNA. From these experiments the investigators concluded that the pseudogenes arose by integration of different mRNA intermediates into the germ-line DNA about 10 and 12 million years ago respectively (Lin and Chan, 1995).

In similar experiments, Weghorst and colleagues (1995) used exon-derived primers to conduct PCR and sequencing to deduce the nucleotide sequence of the pseudogene (Ψgene). They reported a sequence that corresponded to exons 2-11 of the rat p53 cDNA. The sequence was also 87% homologous with the functional p53 gene. In addition, they also characterized two additional putative genes that differed from the functional gene by possessing single and/or double nucleotide substitutions, deletions and insertions. In some cases, these substitution sites also corresponded to mutational hot spots. The authors caution that coamplification of the pseudogene along with the corresponding portions of the functional gene may occur when exon-based primers are used exclusively for PCR amplification. They also caution that

sequence variations found in the pseudogene PCR product could be misinterpreted as somatic cell mutations in an inappropriately generated PCR product mix.

2.2.10 Conserved Characteristics and their Structural Implication

The following characteristics have been reported to be conserved in all the species for which the p53 gene was analyzed so far (Soussi et al, 1990; Soussi and May, 1996).

1. Exon 1 is a noncoding sequence and is part of the 5'untranslated region;
2. A large intron at the 5' end of the gene is present although the significance of it has not been found. The authors also speculate that there could be another gene within the intron.
3. The transcriptional promoter region of the gene is an unusual type in that the characteristic CAAT box, TATA box and G/C rich regions that are normally present in the promoter sequence in most eukaryotic organisms are absent.
4. The only post translation modifications observed in p53 proteins are phosphorylation on multiple serine and threonine residues. Two serine residues at sites 309 and 386 surrounded by two acidic amino acids have been found to be conserved in all the proteins characterized to date.
5. The 100 residues corresponding to the amino terminus of the p53 protein have been were found to consist of a relatively higher number of acidic residues and a very few basic residues. Also, a high proline content has been observed in all the p53 proteins characterized so far.
6. A high charge density with associated hydrophilicity has been observed in the carboxy terminus region of the p53 protein.

7. A hydrophobic region has been observed in the mid-region of p53 that spans 100-300 amino acids and includes domains II, IV and V.
8. In the majority of the cases involving mutations in the p53 gene, characterized in diverse tumors, and in malignant cell lines in mice, conserved regions where the ones that were mutated. This reinforces the hypothesis that the functional domains that are highly conserved are associated with the critical targets of chemical attack and are susceptible to mutations.

2.2.10.1 Crystal Structure of the p53 Protein

p53 is a tetrameric protein. It has multiple domains for DNA binding , trans-activation and tetramerization. DNA binding is sequence specific and resides in the central portion that folds into a compact structure (amino acid residues 102-292). While the transactivation property is confined to the -NH_2 terminal portion (1-44 amino acids), the tetramerization property is seen in the -COOH terminal portion (320-356) (Cho, et al., 1994).

The DNA binding and transactivation regions were reported to be absent in the p53 mutants which indicates that they are critical in maintaining the normal status of the cell. Jeffery and colleagues (1995) reported the crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms using MIR (multiple isomorphous replacement procedure) and has been refined in a crystallographic R factor of 19.2%.

2.3 Ecological and Environmental Importance of Cancer in fish

Estuarine species have been documented to have various neoplastic disorders due to environmental contamination. However, it is often difficult to prove those causes (Harshbarger and Clark, 1990). The reasons for this ambiguity are the presence of chemicals in low concentrations and in complex mixtures. These factors confound the ability to prove any single chemical or a group of chemicals to be the cause of neoplasms or mortality in aquatic species.

Many of the bottom feeding and dwelling fishes such as flounder are in intimate contact with the sediment and their feeding on the invertebrates that themselves feed on contaminated sediments can make them good indicators of estuarine environmental contamination. Sediments can act as good reservoirs of chemicals and hence, potentially increase the exposure of the organism to a chemical (Lyman, 1984). Also, many factors can increase the complexity of interpreting the results of environmental toxicology assessments and include differences in exposure routes, bioavailability of the toxicants and differences in the endogenous metabolism mechanisms of receptor species.

Because of the complexity of such systems many investigators have focused their attention on the identification and validation of relevant biomarkers. Biomarkers are defined as the measurement of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response (McCarthy and Shugart, 1990). This reiterates the fact that it is often very important to obtain data from biological systems in addition to chemical analyses of soil, water and air.

In North America, 41 geographical regions were identified to be the sites where a high prevalence of neoplasms or epizootics of cancer in feral fish have occurred (Harshbarger and Clark, 1990). In these reports, neoplasms were reported in many tissues such as liver, epithelial tissue and pancreas and were also found to show positive correlation with the presence of environmental contamination.

Interest in the study of environmental causes of neoplasms in general was first sparked by an outbreak of epizootic liver tumors in rainbow trout (*Onchorhynchus mykiss*) in mid-1960s in the United States, Japan and several European countries (Halver, 1967). In these countries, the hatcheries were found to have trout feed contaminated with mold metabolites like aflatoxins. In a series of experiments following these large-scale incidents, it was reported that aflatoxins and their metabolites were potent carcinogens and thus could cause liver cancer in many species including man (Halver, 1967).

In Puget Sound unusually high levels of PAHs were found in the sediments and were found to be positively correlated with high incidence of hepatic neoplasms in English sole (Malins et al., 1984, 85, 87, 88). Varanasi and co-workers (1987) studied metabolism of PAHs in various tissues and bile. They studied the distribution, metabolism and covalent binding of these metabolites to the sole hepatic DNA. The results from these studies correlate the presence of PAHs in sediments with prevalence of liver tumors in sole. In additional studies involving analysis of the neoplastic livers of the English sole from Puget Sound, Malins et al. (1990) confirmed the presence of a breakdown product of DNA guanine residue, 2,6-diamino-4-hydroxy-5-formamidopyrimidine.

A similar pattern, but to a more limited extent was observed on the East Coast from Boston Harbor, Massachusetts. Winter flounder were reported exhibiting hepatic neoplasms and a positive correlation was drawn with chemical contamination (Murchelano and Wolke, 1991). In various other studies, neoplasms were documented in populations of brown bull head catfish (*Ictalarus nebulosus*) and Atlantic tomcod (*Microgadus tomcod*). Contamination of the sediment with PAHs was thought to be a causative factor of skin and hepatic neoplasms in brownbull head in the Black River of Ohio (Baumann et al., 1987, 1990). Atlantic tomcod were found to develop hepatic neoplasms after exposure to polychlorinated biphenyls (PCBs) (Klauda et al., 1981). The lesions were also characterized by an increase in the cytochrome P4501A1 gene expression with a concomitant activation of K-ras oncogenes (Wirgin et al., 1989). In additional studies from areas of high industry prevalence near Lake Ontario, the occurrence of PAHs in sediments have been linked to the incidence of hepatic and skin neoplasms of white suckers (Hayes et al., 1990). Suppression of the glutathione S-transferase was observed in white suckers that had hepatocellular and bile duct neoplasms (Stalker et al., 1991).

2.3.1 Fish Models for Environmental Carcinogenesis

The enormous increase in the number of chemicals released into the atmosphere on a daily basis has heightened public interest to know more about the toxicological aspects of the chemicals. While traditional testing using mammalian models are expensive and need to be conducted over long term, results from i*n vitro* tests such as Ames cannot be extrapolated to determine target organ specificity. This has resulted in limited use of *in vitro* tests in bioassays (Goss and Sabourin 1985).

Government regulations indicate that a chemical be released in the market only after sufficient tests have been performed in animal models. In this regard, the use of animals in toxicity testing is a debatable and sensitive issue. Fish species have been identified as alternative models for carcinogenicity testing and have properties (attributes) that are midway to mammalian models and *in vitro* tests. Unlike mammalian models, fish are economical to use and unlike *in vitro* tests can be used to study uptake, distribution and metabolism of chemicals. Also, use of fish in carcinogenicity testing have not yet been criticized thus far. Interest in fish species is pertinent due to the fact that potential human exposure to carcinogens *via* the food chain exists, and because fish can serve as *in situ* field monitors of carcinogenic hazards in ground water near toxic waste sites (Bailey et al.,1996).

Fish occupy a special position in the evolutionary hierarchy among the vertebrates because they are the oldest, largest and are the most diverse group of species (Powers, 1989). Stanton conducted pioneering work in 1965, when he promoted research on using fish as model organisms to study environmental pollution. Other investigators followed this. In their studies they have initiated tumor induction in laboratory experiments to study the mechanisms of tumorigenesis. To correlate some of the laboratory research, field studies were also conducted. However, most of the liver neoplasms that have been reported are in marine and brackish water species (Gardner et al., 1989) and only a few studies have reported neoplasms in fresh water species (Black and Baumann, 1991). In such reports, water pollution due to environmental contaminants (e.g. hepatocarcinogens) has been positively documented to cause cancer in fish (Harshbarger and Clark, 1990; Harshbarger, 1993). In both

laboratory and field, the development of neoplasms in mammalian models and fish has been observed to be similar in most of the organs of fish (Van Beneden et al., 1990). However, for certain organs (e.g., breast, colon, prostate), the difference in structure exists. But due to the fact that tumors are generally graded with the same criteria, histological classification of the neoplasms is possible. These reports indicate remarkable similarity with human tumors (Van Beneden and Ostrander, 1994).

Studies over the past twenty years have focused on the ability of fish to perform a wide variety of biotransformation reactions. Only recent studies focused on the specific metabolites produced due to the biotransformation of xenobiotic chemicals (Lyman, 1984). The distribution, accumulation, and toxicity of chemicals can modify the activity of enzymes that are responsible for the biotransformation process. These reports with certain limitations indicate, fish as attractive alternative models to study the mechanisms of cancer (Farid ,1993). Despite such evidence, not much emphasis is being placed on studying the nature of the disease (cancer) at the molecular level in fish species.

2.3.2 Small Fish in Carcinogenicity Testing

In 1965, Stanton recognized the many positive attributes of the use of aquarium fish for carcinogenesis studies and pioneered the use of fish as surrogates to understand the mechanisms of cancer. He exposed *Zebra danio* to N-nitrosodiethylamine and reported that the fish developed hepatic tumors. More recently small fish are increasingly becoming popular as carcinogenic bioassay models because they are easy to maintain, are economical and very sensitive to a wide range of chemicals. In laboratory studies, fish have been found to develop tumors rapidly (Couch and Harshbarger,

1985; Black, 1984; Powers, 1989; Hendricks 1981; Hawkins et al, 1988 a, b; Dawe and Couch, 1984 and Law et al., 1994). Thus, small fish have been studied as models for predicting and identifying human exposures to environmental contamination (e.g., Fabacher et al., 1991).

In a series of experiments conducted at the Gulf Coast Research Laboratory several small fish species such as medaka, guppy and sheapshead minnow were evaluated as potential bioassay models after exposure to halogenated hydrocarbons. Elaborate aquarium chambers provided chronic exposures of the chemicals at constant concentrations to the fish (Walker et al., 1985). An increasing number of investigators are studying small fish to decipher the mechanisms of cancer (Masahito et al., 1988). Medaka has been a well studied model since it were found to develop retinal medullo-epithelioma, an ocular neoplasm after a single exposure to methylazoxymethanol acetate (MAM-Ac) (Hawkins et al., 1985). In other studies, medaka has also been found to develop exocrine pancreatic neoplasms (Fournie et al., 1987). These initial studies provided the platform for more extensive studies that include oncogene activation in medaka (Van Beneden et al., 1990 and Krause et al., 1997) and DNA repair (Ishikawa et al., 1984). Other investigators have used guppies to study tumor induction on exposure to 7,12-dimethylbenzanthracene and benzo[a]pyrene (Schultz and Schultz, 1982). Law et al., 1994, reported in a study the sensitivity of western mosquito fish to MAM-Ac.

Medaka is often called as the gold standard for carcinogenicity testing. However, it is an exotic species and hence has limited applications in field studies. Among fish, p53 gene has been identified in *Xiphophorous malacatus* (Narin et al., 1996),

Danio rerio (Bailey et al., unpublished results), *Callionymus lyra* and *Platichthys flesus* (Cachot et al., unpublished Results) In an effort to find a native fresh water species that has the above characteristics many investigators have focused their research on the western mosquito fish (*Gambusia affinis*) (Schultz and Schultz1982; Law et al., 1994).

2.3.2.1 The Western Mosquito Fish *(Gambusia affinis)*

The western mosquito fish, *Gambusia affinis*, (Order Atherinifromes; family Poecillidae) is a native of the United States and has wide spread distribution in the country. It is found from New-Jersey to northern Mexico and has also wide spread distribution in the world because it is used for mosquito control (Dees, 1961). It was recognized that these fish could be used as biological agents to control the spread of mosquitoes and hence malaria. When this concept was tested in Camp Augusta, Georgia and proved successful, the International Red Cross asked the U.S government to brood stocks of the fish for other parts of the world, which included Italy and Spain. The fish were found to survive in a wide range of temperature conditions that ranged from 40 ° to 110° F. Their habitats also varied from brackish water to ditches, pools, artesian well discharges, potholes, rain barrels and even sewage outfalls (Dees, 1961).

Many investigators have been successful in rearing these fish in the laboratory using the same techniques as that were used for guppy (*Poecilia reticulata*). The mosquito fish has been well characterized in terms of cytomorphology (Black and Howell, 1979 Campos and Hubbs, 1971). These fish have also been studied for insecticide susceptibility and resistance and have been found to have good genetic plasticity (Ferguson et al., 1966; Vinson et al., 1963; Wise et al., 1986).

Although these fish have been studied for the toxicity of various chemicals (Diamond et al., 1989; Leung et al., 1983; Naqvi and Hawkins 1988) it was not until very recently that neoplastic lesions were reported in these species (Law et al., 1994). Thus, these studies enhance the possibility of mosquito fish as a bioassay model for carcinogenicity testing. More recently Law et al., (1994, 1996, 1997) have studied DNA adduct formation and subsequent tumor induction in *Gambusia affinis* on exposure to methylazoxy methanol acetate (MAM-Ac). Randomly selected 2- to 3-day old progeny of *Gambusia affinis* were exposed to MAM-Ac (10 mg/mL) for a duration of two hours. The fish were moved to grow-out tanks containing fresh water and were sampled periodically to document tumor growth if any. Results were positive in that in as early as 25 weeks after exposure, liver neoplasms were developed in 33% of exposed fish. Within 40 weeks of exposure 52% of the exposed fish developed hepatocellular carcinomas and cholangiocarcinomas. To rule out interference from confounding factors such as spontaneous tumors and infectious conditions, a group of fish were exposed only to clean water for the duration of the experiment (control fish). This group of fish did not show any tumor growth. Comparisons of liver neoplasms of *Gambusia affinis* in this study with liver neoplasms of other aquarium species that have undergone similar kinds of toxic insults indicate a similar carcinogen sensitivity in all of them (Law, et al. 1994).

2.4 Polymerase Chain Reaction and its Applications

2.4.1 History and Applications

The basic method of polymerase chain reaction (PCR) to amplify specific DNA sequences was first invented by Karry Mullis (1985) and reported by Mullis and Faloona (1987). A group in the Human Genetics Department at Cetus Corporation originally applied PCR for analysis of human genetic variation. It has now become an indispensable molecular biology tool that has revolutionized the scientific community in terms of its seemingly infinite number of applications in diverse fields. In any case, detailed discussion of all aspects of PCR and their applications are beyond the scope of this chapter. However, certain aspects of PCR such as the theoretical aspects of PCR, basic methodology and certain milestones in terms of its ingenious applications are detailed below. The procedure has found wide spread use in many areas that include cancer research, genetic counseling for diagnosis of genetic diseases and genetic finger printing of forensic samples, in parentage determination, assays for the detection of infectious agents, in isolation and identification and cloning of novel genes and in fossil studies.

In cancer research its simplicity has led to it being commonly used for detection of mutations and or other abnormalities in DNA, or RNA. The applications of PCR in mutation detection has led to the study of cellular aberrations such as single base pair substitutions, DNA deletions, insertions and translocations. In the field of detection of point mutations, PCR is used to amplify oncogenes or tumor suppressor genes that are then analyzed for point mutations. When gene expression studies are being done using mRNA, a prior step of converting the mRNA to complementary

DNA (cDNA) *via* reverse transcriptase is generally done before conducting PCR. The procedure is commonly called reverse transcriptase polymerase chain reaction (RT-PCR).

The common food borne pathogen *Salmonella* and *E coli* are reported to be the cause of nearly four million cases of outbreaks per year with nearly 1000 resulting in death. These pathogens can cause a wide range of human enteric diseases ranging from gastroenteritis with mild symptoms to typhoid fever to severe debilitating and potentially life threatening illness (Biosystems Reporter, 1996). This has caused great concern about the presence of toxins in food. In response to the food poisoning outbreak the U. S. President, Bill Clinton in 1996 made an announcement regarding the need for regular testing of poultry and meat products for the presence of *Salmonella* and *E coli.*

The current detection methods based on culturing the organism and detection *via* immunological assays, although reliable are time consuming and laborious. Detection of the pathogens can also be done with the help of PCR. This would result in rapid testing for the presence of the pathogen.

2.4.2 The Basic Methodology of PCR

PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to the opposite strands and flank the region of interest in the target DNA. One of oligonucleotide sequence (primer) is upstream of the target sequence and the other is down stream and they are usually 15-30 nucleotide bases long. The whole process is dependent on temperature regulation that plays a critical role in the primer annealing and extension. Also, in

addition to the target DNA, it is important to add appropriate buffers, salt (usually magnesium chloride), deoxynulceoside triphosphates and a thermostable DNA polymerase such as Taq polymerase.

The simplicity of the PCR reaction has mainly stemmed from the discovery of the thermostable Taq polymerase and the automation of the temperature cycling (Erlich et al., 1990). In the initial stages of development of PCR, the Klenow fragment of *E coli* DNA polymerase I was used. This enzyme was inactivated during the heat denaturation step in each cycle, when the two stands of DNA were separated. Consequently, fresh enzyme had to be added to each cycle to maintain the enzyme level. Taq Polymerase isolated from *Thermus aquaticus* was found to withstand repeated exposure to the high temperature (94 °C - 95 °C) required for strand separation. This discovery transformed the reaction into a simple and robust method. Starting from minimal amounts of DNA, (as low as one molecule) large amounts of the known sequences can be generated. The procedure is very versatile in that, it works well when the starting material is clean and also for situations when the starting material is in a crude form. The whole process consists of 3 steps that include template denaturation, primer annealing, and extension of the annealed primers by the DNA polymerase. The first step involves heat denaturation wherein, the two strands of the targeted DNA molecule separate. In the second step, the separated strands of DNA base pair with small oligonucleotide sequences (primers) of synthetic DNA that have sequence information that complements with one strand of the target DNA. In the last step, the primer serves as a starting point for extension of the nucleotides

complementary to the target DNA. These three steps are repeated 25-35 times to obtain an exponential increase in the target DNA.

2.4.3 Theoretical and Practical Aspects of PCR

2.4.3.1 The Exponential nature of PCR

PCR is a chain reaction wherein, the products of one cycle of amplification act as substrates for the next cycle. For that reason, the amount of product increases exponentially and not linearly as in most enzymatic reactions. Under ideal conditions, the amount of product doubles during each cycle according to the following equation:

$$N = N_0 2^n \qquad \text{(Eqn. 1)}$$

where,

N = The number of amplified molecules, N_0 = the initial number of molecules and n = the number of amplification cycles

2.4.3.2 The Efficiency of Amplification

The efficiency of amplification (E) can be defined as the fraction of template that is replicated during each reaction cycle. Experimentally it is less than perfect. It can be described by the following equation:

$$N = N_0 (1 + E)^n \qquad \text{(Eqn. 2)}$$

Due to the exponential nature of the PCR, a small change in the amplification efficiency (E) can change the amount of the product N, dramatically even if the initial number of target molecules N_0 is the same. A few factors that affect the efficiency of the amplification process include the actual sequence being amplified, its length, and the sequence of the primers and presence of impurities in the sample. The success or failure of a PCR reaction is largely dependent on the selection of primers. There are a

few guidelines that must be followed for primer selection. Primers must have a random base distribution with a GC content similar to that of the target DNA. Stretches of polypurines and polypyrimidines are generally avoided. It is also prudent to avoid secondary structure at the 3' end of the primer. Last but not the least is the necessity to check the two primers for complementarity to avoid primer-dimers. Impurities in the sample can decrease the amplification efficiency by degrading the polymerase and causing conformational changes in the target DNA. Impurities can also compete for the primer binding sites and affect the amplification efficiency.

2.4.3.3 The Plateau Effect

Experimentally, the amount of amplification product in a PCR increases exponentially only until a certain point after which it decreases and finally levels off. The leveling of the rate of amplification is called the plateau effect. A few factors that can affect the time to reach the plateau effect are the molar ratio of the polymerase and the template, the product reassociation, competition by non-specific products, and one or more of the components of the reaction can become limiting. The number of cycles that need to be completed before plateau effect also varies from reaction to reaction and is also dependent on the target DNA sequence. Once plateau occurs, increasing the number of cycles will not increase the amount of the product. This factor has to be determined experimentally by varying the number of cycles and visualizing the product.

2.4.3.4 Deficiencies

The ability of PCR to generate large amounts of DNA product from minute amounts of DNA can give rise to an undesirable draw back in that amplification of

false positives can occur from sources other than the template in the reaction. The sources of contaminating DNA can be varied. A few examples of such sources for contaminating DNA are from other templates that were handled in the laboratory, or that were generated from a previous amplification or contamination due to carry over. This last method of contamination is very troublesome and difficult to control. Prevention of carry-over is generally advised as opposed to controlling it. To prevent carry-over contamination the reactions should be set up in a separate room or biosafety containment unit and separate set of supplies and pipeting devices should be maintained for the pre- and post PCR amplifications. Positive displacement pipettes should be used and uncapping the tubes carefully should prevent aerosolization of PCR products and templates. Carry-over contamination can be prevented to a large extent when the pre and post PCR amplification procedures are separated. Carry-over is also observed if the reagents are not aliquoted in smaller volumes and repeated sampling is done from the same vial and if the reagents and primers are not stored in areas that are free of the PCR amplified product. It is also prudent to change gloves frequently and to include negative controls that do not contain any template as a means to monitor carry-over contamination.

In situations, wherein there is concern that carry–over contamination has occurred, certain procedures exist that can help eliminate the problem. A few of these are ultraviolet irradiation of surfaces and reagents, (Cone et al., 1990) and enzymatic control with the help of nucleases. UV radiation results in formation of cyclobutane rings between the neighboring thymidine or cytidine. These result in the formation of intrastrand pyrimidine dimers that inhibit polymerase-mediated elongation (Cone et

al., 1990). However, UV irradiation of laboratory surfaces is often a cumbersome task because the surface must be perpendicular to the light source to achieve optimal intensity. For a more robust method incubating the amplification reaction with the enzyme UDG results in removal of dU from carry-over DNA, but does not affect DNA, dUTP or RNA. This results in the creation of many abasic sites that can stall DNA polymerase.

2.5 Methylazoxymethanol Acetate

Methylazoxymethanol acetate (MAM-Ac), is derived from cycasin and is labeled as an experimental carcinogen and a teratogen. MAM-Ac can be extracted from the roots, leaves and seeds of cycad plants. MAM-Ac is also a by-product of the breakdown of a rocket fuel (Personal communications Ostrander G. K). The molecular formula and molecular weight of MAM-Ac are $C_4H_8N_2O_3$; and MW: 132.14. It has gained acceptance as an experimental carcinogen that requires metabolical activation.

The toxicity of the MAM-Ac is basically liked to methylazoxymethanol (MAM) that has the following molecular formula: $C_2H_6N_2O_2$. This is a relatively unstable aglycone of cycasin. Cycasin is a naturally occurring and needs to be extracted from the roots, leaves and seeds of cycads. The cycads belong to the family cycadaceae, are gymnosperms and have wide spread occurrence in tropical and subtropical regions of the world (Laquer et al., 1963). Among the cycads, two species, *Cycas circinalis* and *Cycas revoluta*, have been widely studied due to their epidemiological association with neurological diseases in areas where these plants were consumed.

The seeds are rich in starch and were consumed by the native populations in times of famines and typhoons, when the normal food sources were scarce. However, Laquer and co-workers in the year 1963 reported that the natives knew of the toxicity of the seeds and therefore, took precautions such as dehusking, slicing or repeated washing of the seeds in an effort to detoxify and make the flour of the seeds safe for consumption. Inadequate treatment led to death of many individuals (Spatz, 1969).

Cycasin was extracted from *Cycas revoluta* by Nishida et al (1955). They reported the glucoside to be toxic to mice and guinea pigs when administered enterically. Nishida and colleagues attributed its toxicity to the aglycone moiety. The hydrolysis of the parent compound was reported to occur in the digestive tract of the experimental animals. Reports from carcinogenicity tests performed on the cycasin fractions revealed extensive liver damage in acute toxicity tests and in chronic toxicity tests hepatomas were observed (Matsumoto and Strong, 1963). Matsumoto and Higa synthesized MAM-Ac in the laboratory (1966). The metabolic studies conducted for this compound indicate that both the natural compound cycasin and MAM-Ac, the synthetic compound are metabolized to MAM which is a relatively unstable and reactive species. Nishida et al., (1955), reported the hydrolysis of cycasin as follows:

Gl- $C_2H_5O_2N_2$ + H_2O = Glucose + N_2 + HCHO + CH_3OH ; (Where *Gl* is the glucosyl residue).

While cycasin administered through oral routes was toxic to animals, MAM was reported to be toxic when administered by all routes. β-glycosidase present in the cells of the small intestine and in the bacterial flora of the large intestine were reported to be responsible for converting cycasin to MAM. An interesting discovery was made

regarding the theory that cycasin is toxic only by the oral route due to the presence of β-glycosidase in intestine that could breakdown cycasin to MAM. Rats only in the early postnatal days (3-4 days) were susceptible to cycasin administration and not later on. The initial explanation for the toxicity was attributed to the fact that the dam's on cleaning their young ingest cycasin, that then gets metabolized to MAM and reaches the pups through the dam's milk. However, when artificially nursed pups displayed the same susceptibility, another mechanism was thought to be present. In a series of experiments following this discovery, β-glycosidase capable of hydrolyzing cycasin to MAM was isolated in the skin of new born and early postnatal rats. Such transient biological enzyme systems can provide the basis for toxicity studies in neonatals.

MAM-Ac was studied by many investigators in small aquarium fish (Stanton, 1965; Hawkins et al., 1985; Law et al., 1994). Most of the investigators reported hepatomas and liver tumors in their experiments. Harada and Hatanaka (1988) reported esinophilic foci, basophilic granules and also changes in mitochondria and endoplasmic reticulum as early as two weeks of post exposure to MAM-Ac. In primary hamster embryo cells, this compound was found to increase the expression of the oncogenic simian adenovirus7 by 3-8 fold (Casto et al., 1974)).

More recently, Law et al., (1994) based on the earlier studies exposed *Gambusia affinis* to MAM-Ac to induce hepatic tumors and also study p53 expression in control versus exposed fish. They were successful in that they were able to induce tumors in 24 weeks of exposure.

The general consensus of many investigators is that MAM-Ac can produce observable effects in a relatively short time. Also, MAM-Ac is labeled as complete

carcinogen, which indicates that it has both tumor initiating and promoting ability (Hawkins et al., 1985). As such a single dose was in many cases sufficient to produce tumors in test animals.

2.6 Polycyclic Aromatic Hydrocarbons

Organisms are continuously being exposed to the toxic properties of a large suite of contaminants either through food, water or soil. These chemicals may be either natural products or anthropogenic in origin. Large amounts of chemicals are released into the atmosphere, water and soil during the process of production of chemicals during usage and/or disposal of the chemicals. The extent of chronic exposure to low levels of carcinogens resulting from these processes and the potential role of such exposures is an area of concern to health authorities (Rall, 1990).

Polycyclic aromatic hydrocarbons (PAH) are a group of compounds that constitute the biologically active and significant portion of the hydrocarbons that initiate carcinogenesis after metabolic activation. PAHs are present ubiquitously in the aquatic and terrestrial environments (Eisler, 1987). The correlation between exposure to chemicals and the development of cancer was first documented by Percival Pott in 1775, when he observed that chimney sweepers exposed to soot developed scrotal cancer. Later on, experimental studies were initiated by a group of Japanese investigators, Yamagawa and Ichikawa in early 1900's to study the carcinogenicity of the coal tar. In their studies, they synthesized dibenz[a,h]anthracene. Later on, Cook et al. (1933) while studying the carcinogenicity of coal tars discovered benzo[a]pyrene (B[a]P).

Aquatic sediments are invariably major repositories of contaminants such as PAHs introduced into surface waters (Lyman, 1984). These chemicals sorb on the sediment resulting in concentrations that are several orders of magnitude higher than the concentration of the chemicals in water (Lee and Jones, 1984). Benthic organisms are continuously being exposed to high levels of PAH as they are in intimate contact with the sediments (McElroy, 1985; Varanasi and Gmur, 1980). In addition to being toxic to the aquatic life, contaminated sediments by themselves can act as a source of contaminants for bioaccumulation in the food chain (Lee and Jones, 1984). PAHs are lipophilic and are converted to more water-soluble forms through metabolic activation such as reduction, oxidation, hydrolysis or conjugation that facilitate their excretion. It is thus, important to study the availability of PAHs to benthic organisms, their remobilization from the sediment reservoirs, the ability of the organisms to metabolize and excrete PAH, and finally the ability for potential transfer between aquatic and terrestrial organisms.

Cytochrome P-450 enzymes that will either lead to activation or inactivation of the compounds catalyze transformation of many endogenous compounds (steroids) and exogenous compounds (pollutant chemicals). The difference in the metabolic pathways of various species might be due to the presence of different complements of P-450 proteins, and also due to the differences in their catalytic function and regulation. Knowledge of the susceptibility of toxicity of various species due to biotransformation is also important.

The cytochrome P-450 mediated monooxygenase system evolved as a detoxification system to convert non polar PAHs to hydroxylated primary intermediates.

These primary metabolites are then conjugated with glucuronide or glutathione to facilitate their excretion. However, for many xenobiotics such as PAHs, the enzyme system can act in a paradoxical fashion and catalyze the oxidative formation of reactive intermediates. These reactive intermediates can act as electrophiles that can covalently bind to DNA and cause mutations (Varanasi et al., 1987, Hall and Grover, 1990).

Metabolic activation of xenobiotics has been an active area of research in aquatic toxicology and will not be discussed in this chapter. However, very little emphasis is directed towards understanding the molecular basis of carcinogenesis in aquatic organisms. Among fish, p53 to date has been characterized only in trout (Soussi et al., 1992; Kusser et al., 1994) and in medaka (Krause et al., 1997). It therefore becomes important to identify and characterize tumor suppressor genes in fish to elucidate the mechanisms of cancer formation in fish (Van Beneden et al., 1990).

2.7 PAHs and their Involvement in p53 Gene Inactivation

A high frequency of G to T transversions in the p53 gene were observed in tobacco related neoplasias like small-cell and non-small cell lung cancers (Takahashi et al., 1991), esophageal carcinomas and squamous cell carcinomas of head and neck (Burns et al., 1991). The presence of specific mutagens such as B[a]P and related compound in tobacco smoke are implicated in the observed mutagenicity. Ruggeri et al. (1994) conducted *in vivo* studies in mouse to document the frequency and pattern of p53 mutations on exposure to B[a]P. They reported B[a]P species to react with guanine residues leading to adduct formation and a high frequency of G to T

transversions. The mutations were always documented to occur in the hot spot regions of p53, the tumor suppressor gene. Skin tumors were induced in mouse with B[a]P. Genomic DNA was extracted from the tumors and polymerase chain reaction (PCR) was conducted to amplify the p53 gene. Direct DNA sequence analysis was conducted in regions spanning exons 5-8 as they spanned the conserved regions and thus, the hot spots. It was reported in their study that guanine residues were the main targets with majority being heterozygous mutations in exons 7 and 8. Of the mutations that were reported, 70% were G to T transversions. In a more recent study by a group of investigators at the John Hopkins center, a direct link between B[a]P and lung cancer was reported (Denissenko et al., 1996).

Ruggeri and colleagues (1991) in some earlier work with dimethyl benzanthracene (DMBA) reported a similar pattern of mutations although the frequency of mutations was much lower. A two step protocol of exposure to 7,12 DMBA or a complete carcinogenesis protocol resulted in a lower frequency of mutations (30%) when compared to B[a]P induced mutations in the p53 gene. This study reiterates the fact that the p53 gene may be a selective target for different carcinogens and the metabolically activated B[a]P may be responsible for human tobacco related neoplasias.

Mutations in the coding region of the hypoxanthine (guanine) phosphoribosyltransferase (HPRT) gene have also been studied after B[a]P exposure in Chinese hamster V-79 cells (Caroline, et al., 1991). In their studies the authors report that high doses of exposure to B[a]P, caused base substitutions exclusively at the G-C pairs whereas, lower doses caused substitution mutations at both G-C and A-T base pairs.

This study is important in distinguishing the dose dependent differences in the mutational spectra with B[a]P exposure.

Kawajiri et al. (1996), studied the association of P450 1A1 (CYP1A1) germ line polymorphisms with the mutations in the p53 gene in lung cancer cases. The frequency of mutations in heavy smokers was higher than in patients who never smoked. Also, it was reported that smokers with a genetic disposition for susceptible rare homozygous alleles of the CYP1A1 gene to be more susceptible than the ones with nonsusceptible homozygous alleles.

2.8 Sequencing with PCR for Mutation Detection

The applications of PCR are growing exponentially similar to the theoretical nature of the amplification process (Erlich, 1990). The ability to amplify DNA from fresh tissues, frozen tissues, and /or paraffin embedded tissues has resulted in major advancements in mutation detection technology. The reason for the progress in detection of mutations from tumors stems from the fact that very small amounts of starting material are all that are required for amplification by PCR (Malarkey and Maronpot, 1996). Protooncogenes and tumor suppressor genes are involved in the important cellular mechanisms that regulate growth and differentiation. Mutations in these genes can trigger a series of events resulting in the formation of tumor. Screening for mutations such as translocations, insertions or deletions have been conducted traditionally with Southern blot analysis and filter hybridization of enzyme digested DNA. However, detection of point mutations by these procedures is not feasible. The limitations for using Southern hybridization for mutational analysis are that mutations must be

present in at least 1% of the cell population and large amounts of the genomic DNA should be available. This is not possible in all cases. In certain situations, DNA is partially degraded and embedded in paraffin as is the case of tumor samples wherein pathologists routinely section and preserve tumor tissues. With the advent of powerful techniques such as PCR, the possibilities exist for revisiting the samples to obtain additional information regarding the nature and cause of the tumor. The procedure of isolating DNA from paraffin embedded tissues and other archived samples for use in molecular biology techniques such as PCR and sequencing is termed retrospective analysis (Shibita et al., 1994).

PCR in conjunction with a few mutational assays such as single-stranded conformational analysis (Orita, 1989), Restriction endonuclease digestion, Allele specific oligonucleotide hybridization (Saiki et al., 1988) and direct sequencing (Sanger et al., 1977) has resulted in improving the ability to detect mutations. It is not the purpose of this chapter to include the methods as some excellent review articles are available for mutation detection assays (Malarkey and Maronpot, 1996; Loda, 1994). Mutations detected by the various methods, need to be subsequently confirmed by direct sequencing. In addition, sequencing in conjunction with amplification by PCR itself is a procedure for detection of base substitutions of oncogenes and or tumor suppressor genes. Advances in sequencing technology that include automation of the process, use of fluorescent labeling, and cycle sequencing with Taq Polymerase have all resulted in the ability to directly sequence double-stranded DNA *via* production of dideoxy-terminated sequences (Loda, 1994) without the need for generating single stranded DNA by assymetric PCR.

Chapter 3

Materials and Methods

The effects of chemicals are frequently investigated on organisms undergoing long term exposures (2-12 months) and the visual changes that may result from these exposures such as neoplasia, hyperplasia and dysplasia are studied using histopathology and or immunochemistry. It is increasingly being accepted that the molecular events that occur in an organism during the early periods of exposure to chemicals are equally important. Environmental pollutants can potentially cause damage to DNA that can be evaluated in terms of changes in gene sequences and or gene expression and used as biomarkers to interpret the genotoxic properties of chemicals (Kahn, 1983). Most of the studies of p53 inactivation by PAHs such as benzo[a]pyrene were conducted in mammalian models and very little emphasis was given to lower vertebrates like fish. The ubiquitous presence of PAHs such as benzo[a]pyrene in the aquatic environment coupled with their well-documented mutagenic and carcinogenic properties warrants the need to study the effect of these compounds on the p53 gene in fish species. Although many investigators reported the use of *Gambusia affinis* in bioassay studies, information pertaining to the molecular aspects of the cells (gene sequences and gene expression) has not been reported. Prior to conducting any mutational studies using *Gambusia affinis*, it is necessary to characterize the wild-type sequence of the p53 gene in *Gambusia affinis*. The main objective of this project was the identification and isolation of the p53 gene DNA sequence corresponding to exons 5-8 of the Western mosquito fish (*Gambusia affinis).*

Polymerase chain reaction (PCR) (Mullis and Faloona, 1987) is fast becoming an indispensable tool for molecular toxicology. It is a relatively simple technique to efficiently amplify specific DNA sequences to detectable and analyzable levels even from small to miniscule amounts of crude starting material. However, optimization of the reaction is dependent on many variables, which primarily include: purity and concentration of the nucleic acid, magnesium chloride concentration and annealing temperature of the primers. For this project, PCR was the preferred procedure to isolate and characterize p53 gene in *Gambusia affinis*. Adult *Gambusia affinis* measure only about 3-5 cm in length. To increase yield of nucleic acids (DNA/RNA), adult *Gambusia affinis's* livers were pooled for extraction of nucleic acids. This section mainly describes protocols that were adapted for *Gambusia affinis* after modification of the standard procedures (Sambrook et al., 1989). These include: DNA and RNA extraction from livers of *Gambusia affinis*, optimization of PCR using a GeneAmp™ PE9600 (Perkin Elmer Corporation, Norwalk, CT), Reverse-Transcriptase PCR to generate a cDNA, cloning of PCR products using the Original TA Cloning Kit® (Invitrogen Inc, Carlsbad, CA), and fluorescent sequencing of PCR products and cloned products using an ABI PRISM™ 310 sequencer (Perkin Elmer Corporation, Norwalk, CT). In addition, Southern hybridization followed by chemiluminiscent detection was conducted to confirm the presence of the p53 gene in *Gambusia affinis*. In the latter part of the section, screening for mutations was conducted by comparing DNA sequence of exons 5-6 of *Gambusia affinis* exposed to MAM-Ac with that of the wild-type sequence.

Three month old specimens used in the study were kindly provided by Dr. Hawkins of the Gulf Coast Research Laboratories, Mississippi and were acclimatized in the Aquatic Laboratory Facility, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA. The rooms for holding fish were adjusted for maintaining 16-hr light/8 hr dark cycle with fluorescent lighting. The fish were acclimatized by substituting the water in the bags with dechlorinated water that was maintained at pH 8.4, hardness 250 mg/L and at a temperature of 80° F over a period of 2-3 hours. About twenty-five fish were placed in 20-gallon tanks that were equipped with air and charcoal filters. Fish were fed commercial flake food (Prime Tropical Flakes –Yellow, Zeigler Brothers, Inc., Gardners, Pennsylvania) twice daily. Tank maintenance and water quality monitoring were conducted once a week to remove any material accumulated in the tanks.

3.1 Sequencing of the p53 Gene in *Gambusia affinis* in Exons 5-8

In this section, two approaches are described to isolate the sequence corresponding to exons 5-8 of *Gambusia affinis*. In the first protocol (DNA-PCR), genomic DNA was extracted from the livers of *Gambusia affinis* and PCR followed by sequencing was conducted. In the second protocol, total RNA was extracted from the livers of *Gambusia affinis* and Reverse–Transcriptase-PCR (RT-PCR) was performed to generate cDNA. Figure 3.1 is a schematic representation of the two approaches.

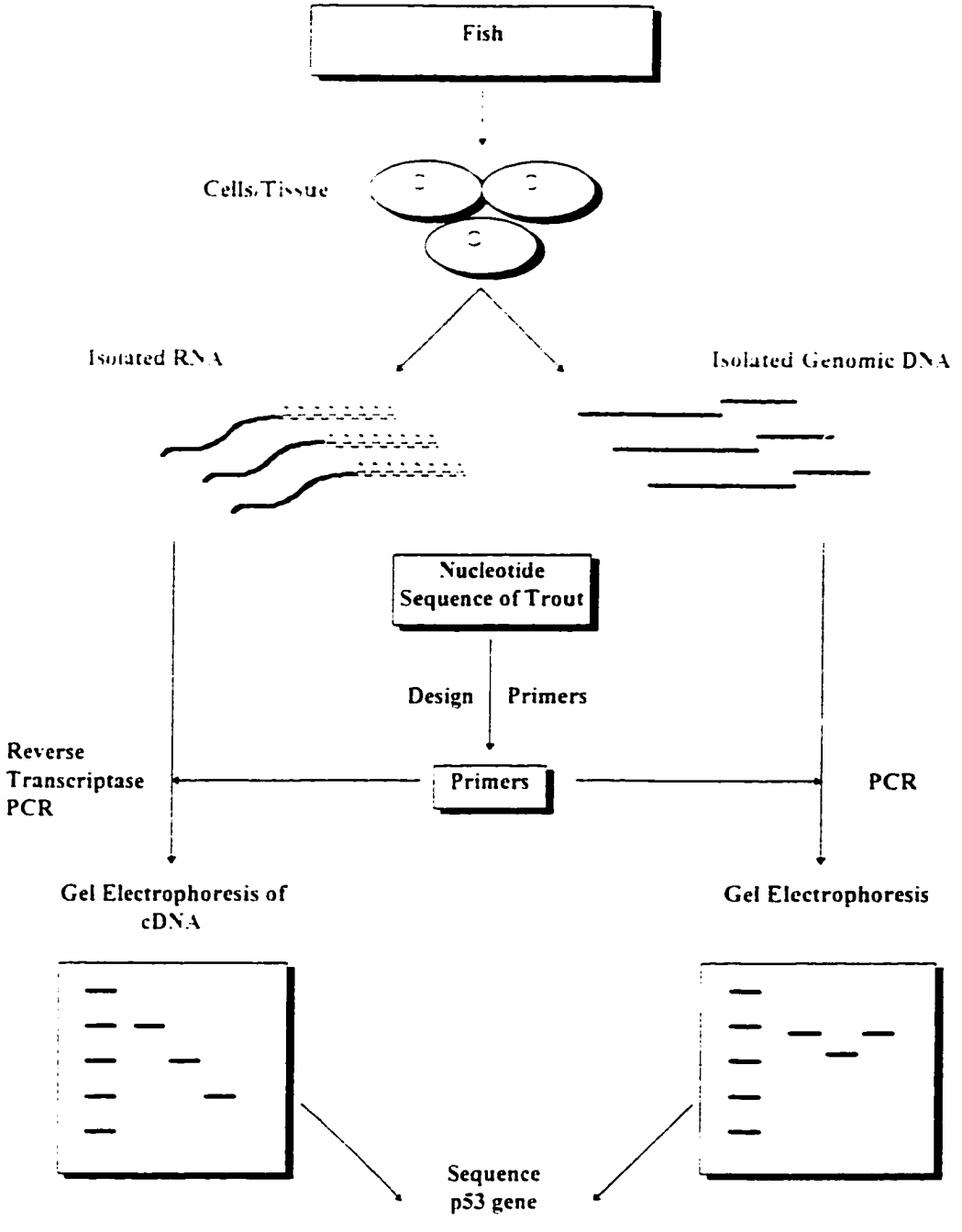


Figure 3.1 Schematic representation of the approaches followed

3.1.1 DNA –PCR

3.1.1.1 DNA Extraction and Purification

Genomic DNA was extracted from *Gambusia* affinis according to standard procedures described in Current Protocols (Sambrook et al., 1989) with some modifications and are described in the following paragraphs. Five fish were randomly selected and euthanatized by placing on ice. Livers were immediately excised from each fish, pooled together and the weight recorded. The pooled liver was immediately transferred to tightly sealed sterile 1.5 ml microcentrifuge tubes (~110 mg per tube), and frozen by briefly placing in liquid nitrogen tanks. The tissue was then pulverized in 2 ml polypropylene microcentrifuge tubes and suspended in digestion buffer (1.2ml/110mg of tissue) to which proteinase K (final concentration 0.1 mg/ml) was added. Proteinase K being labile must be added fresh for each extraction. The samples were incubated with shaking at 50 °C for 12 to 18 hr. The digested samples were extracted with equal volumes of phenol: chloroform: isoamylalcohol (25:24:1, pH 8.0, Amresco, Solon, Ohio, USA) and centrifuged at 1300 x g for ten min. This step allows the separation of aqueous and lipid phases. DNA is present in the aqueous phase and was precipitated by the addition of isopropanol and 7.5 M ammonium acetate followed by centrifugation at 1300-x g for two min (to reduce residual RNA). DNA forms a pellet at the bottom of the microcentrifuge tube, which was rinsed with 70% ethanol and briefly air-dried. The pellet was finally suspended in deionized water and shaken gently at room temperature and placed at 65 °C for several hours to facilitate solubilization. To determine the concentration and purity of the genomic DNA, an aliquot of the DNA solution (1:200 dilution in water) was used to measure the

absorbance at 260 and 280 nm using a Beckman DU-600 Spectrophotometer according to the two wavelength method of Warburg and Christian (Layne, 1957). Aliquots of the DNA were stored at –20 °C and working concentrations (10 ng/ μL) of the same were stored at 4 °C. Electrophoresis of an aliquot of the DNA was conducted to check for purity and degradability.

3.1.1.2 PCR Amplification

- **Equipment and Reagents for PCR**

All experiments were performed using the GeneAmp PCR system 9600 according to manufacturer's instructions (Perkin Elmer Corporation, Norwalk, CT). All PCR reagents were obtained from Perkin Elmer / Applied Biosystems, Foster City, CA. Primers ranging in length from 20-24 bases were selected from the cDNA sequence of rainbow trout after identifying regions of homology in other species and tested for compatibility by using the Oligo 5.0 ^{Tm} program. Primers from the intron regions were obtained from the p53 gene sequence of rainbow trout in exons 5-8 (Kusser et al., 1994). Primers were synthesized on a Perkin Elmer /Applied Biosystems DNA synthesizer Model 394 at the Gene Lab at Louisiana State University, School of Veterinary Medicine, Baton Rouge, LA, and in other cases were also obtained from Genosys Biotechnologies Inc., Woodlands, Texas. The primer combinations are listed in Table 3.1.

The standard PCR parameters were followed in most cases, with certain modifications. For each set of primers, hot start conditions were applied according to

Table 3.1 Primer combinations for exons 5-8 from Rainbow Trout cDNA used for Gambusia affinis

Exon/Intron	Forward Primer(5'- 3')	Melting Temperature	Exon/Intron	Reverse Primer (5'- 3')	Melting Temperature
Exon 5	NH1 (CAGTTTTTGGCGAAGACTTGTCC)	66°C	Exon 6	NH2R (GGCTCATAGGGGACGG AGCAC)	70°C
Exon 5	C1 (TACTCGCCAGACCTGAACAAGT TG)	62°C	Exon 5	GR1 (GGTGAGGGCAGCGTCT CA)	62°C
Exon 5	C1 (TACTCGCCAGACCTGAACAAGT TG)	62°C	Intron 5	I5R (TCAAAGCACATTCTAG A)	62°C
Exon 7	NHF7 (CAACTTCATGTGCAACAGCTC)	62°C	Exon 8	NJBR1 (AAACTCCACGCACACA CGGACA)	62°C
Exon 7	NH3 (CCATCCTCACCATCATCAACC)	62°C	Exon 8	NJBR1 (AAACTCCACGCACACA CGGACA)	62°C
Exon 7	NH7F (GTGGGATCAAGAGTGTACCCAC)	62°C	Exon 8	NJBR1 (AAACTCCACGCACACA CGGACA)	62°C

the manufacturer's recommendations (Perkin Elmer / Applied Biosystems, Foster City, CA). The composition of the lower (40 µl) and upper reaction mix (60 µl) were as follows: lower reaction mixture contained 10 x PCR buffer II) (2.5 µl) without magnesium chloride, 25 mM magnesium chloride (Part No.N808-0010) with a final concentration in the range of 1.5 – 4 mM, 200 µM of each dNTP (Part No. N808-007) and 0.4µM of each primer (forward and reverse) and sterile water. The reaction mixture was heated at 80°C for 5 min. The upper reaction mix contained the remaining amount of water, 2.5 Units of Taq Polymerase™ and genomic DNA (10 –30 ng). The thermal cycler was let to cool to 25 °C. Amplification reactions were set for 30-35 cycles with 96° C for 45 sec, 65 ° C for 1 min and 72° C for 2 min. A final incubation for 10 min was included for extension of unfinished products. Annealing temperature was varied depending upon the melting temperature of the primers in 2°C increments until the desired band was produced. For certain regions of the p53 gene that were hard to amplify the Hot Wax OptiStart™ Kit (K1420-01, Invitrogen Corporation, Sorrento Valley Blvd. SanDiego, CA) was used. The kit includes four PCR buffers at different pH levels (8.5-12) in tandem with various salts (e.g., ammonium sulfate and potassium chloride) and is provided by the manufacturer without magnesium chloride. Three levels of magnesium chloride (1.5, 2.5 and 3.5 mM) are supplied coated on color-coded wax beads to provide hot start for the reaction mixture. In this way, a 4 X 3 matrix (total of 12 combinations) resulted (Table 3.2) that helped in determining the optimum signal strength and specificity for each PCR product.

Table 3.2 4 X 3 matrix of four different pH levels of PCR buffer and three different levels of Magnesium Chloride coated on HOT WAX™ beads

5 X PCR BUFFER	HOT WAX™ BEAD $[Mg^{2+}]$ IN mm (1X)		
	1.5	2.5	3.5
pH 8.5	1	2	3
pH 9.0	4	5	6
pH 9.5	7	8	9
pH 10.0	10	11	12

to minimize pipeting variables, a master mix containing all the reagents was made and an equal volume was added to each tube. Extreme care was taken to prevent carry-over contamination. For this purpose, a separate room was maintained to add all the reagents other than the template. Positive and negative controls were included in all cases. Rainbow trout genomic DNA was used for the positive control at a low concentration (5ng), so that a weak but consistent band was obtained with all the primer combinations. All the reagents except DNA were added for negative control and sterile water was substituted in place of genomic of DNA. Primers and dNTPs were aliquoted in smaller volumes and stored at –20° C to prevent repeated freezing and thawing of the samples. Sterile surface area (Centrifuges and thermal cycler) was always maintained by cleaning with ethanol and 10 % chlorox. Positive displacement pipets and aerosol-filtered tips were used to set up the reactions. The reagents were UV irradiated by placing them on the UV light box for 5 min. (Cone et al., 1990).

3.1.1.3 Agarose Gel Electrophoresis and Detection

The process of electrophoresis is based on the fact that nucleic acids are uniformly negatively charged and the double stranded DNA is free of complicating structural effects that affect its mobility (Sambrook et al., 1989). It is necessary to review the various factors that affect the migration of the nucleic acids on gels. These include the conformation of the nucleic acid, the size of the pore, the voltage gradient applied and the type and concentration of the buffer. The pore size is an important variable as it can determine the size of the nucleic acid that has to be separated by electrophoresis. Large fragments (500-1000 base pairs) can be resolved on larger-pore agarose gels, whereas, small fragments (less than 1000 base pairs) can be

resolved on smaller-pore acrylamide gels. Nuseive®3:1 agarose (FMC BioProducts, Rocklane, ME) was used to conduct electrophoresis of DNA. This mixture of agarose is standard gelling (32.8-38 °C) and has melting temperature of (< 90° C). This mixture of agarose when made up in 1X Tris acetate buffer has the capacity to resolve nucleic acid fragments less than 1000 base pairs and can also distinguish fragments as small as 10 base pairs. This agarose has low viscosity and when used at high concentrations can be used to produce high-resolution gels. A 2% Nuseive^{R} (3:1 Agarose, Order No. 50092; FMC Bio Products, Rockland, ME) was prepared by dissolving 2g of Nusieve (3:1) in 100 ml of 1XTris acetate buffer (40mM Tris acetate, pH 8, 2mM EDTA) (Amresco Solon, Ohio, USA) by heating in a microwave. After the solution had cooled to room temperature, it was poured onto a tray and allowed to set. The tray was transferred to an electrophoresis chamber (GIBCO-BRL Life Technologies, Gaithersburg, MD.) containing 1X TAE. For analysis, 10 µl of PCR product and 2 µl of loading dye (Amresco, Solon, Ohio) were loaded in the wells and electrophoresis conducted at 80 Volts for 1hr and 45 min. 4 µl of Low DNA Mass ladder (Cat. No 10068-013) and 12 µl of the 123 base pair DNA ladder (Cat. No. 15613-029) both from GIBCO-BRL, Life Technologies Gaithersburg, MD were used as DNA size standards. For detection, the gel was immersed in ethidium bromide (5 µg/ml) for 5 min and then transferred to a UV transilluminator (Fotodyne Inc.,) at 302 nm.

3.1.1.4 Cloning PCR Products

The pCR II™ and pCR 2.1™ vectors that were included in the Original Cloning kit ® obtained from Invitrogen Inc., (Carlsbad, CA).

- **Production and Detection of PCR products**

For the first step a PCR product was generated and analyzed by electrophoresis according to the procedures described in section 3.1.12. The amount of DNA was quantified by checking the absorbance at 260 and 280 nm for each PCR product.

- **Cloning of PCR products into pCR ™2.1 vector**

The ligation reaction in a final volume of 10 µl contained the following :

Sterile water	5 µl
10 x Ligation Buffer	1µl
pCR ™ 2.1 vector (25 ng/µl)	2µl
Fresh PCR product (~10ng)	1 µl
T4 DNA Ligase	1µl

The ligation mixture was mixed gently and incubated at 14° C overnight. The samples were removed the next day, briefly centrifuged and placed on ice until transformation.

- **Transformation**

An Appropriate number of One Shot™ cells was thawed on ice. To this, 2 µl of 0.5 β-mercapto ethanol (β ME) was pipetted and added to the cells on ice. The mixture was mixed gently with the pipette tip. 2 µl of the ligation mixture was pipetted into the cells and stirred gently with a pipette tip. The vials were incubated on ice for 30 min, followed by heat shock for exactly 30 sec at 42 °C and the vials were placed again on ice for an additional 2 min. To each vial 450 µl of SOC medium was added

and the vials were shaken at 37 °C in a shaker at 225 rpm for 1 hr. The vials with the transformed cells were placed on ice for analysis.

- **Analysis**

To screen for white and blue colonies, 50 µl and 200 µl of the transformed cells were plated on LB plates with 50 µg/ml ampicillin and 40 µl of IPTG. The plates were incubated at 37° C overnight and checked for colony growth the next morning. White colonies if any, were picked and sequenced using T7 primer to confirm sequence of the PCR product (insert).

3.1.1.5 Sequencing of PCR products

PCR products were sequenced using the ABI PRISM™ Dye Terminator Cycle sequencing Ready Reaction Kit with AmpliTaq^{R} DNA polymerase FS (Perkin Elmer Corporation, Norwalk, CT). In situations, where a single PCR band was obtained, the PCR product was purified of unwanted primers, and impurities such as salts, enzymes and unincorporated nucleotides, using QIAquick™ kit (Qiagen Inc. De Soto Avenue, Chatsworth, CA.). This procedure is a rapid and convenient technique that incorporates a microspin technology coupled with a specially adapted silica-gel membrane that can bind nucleic acids. DNA gets adsorbed onto the silica gel in the presence of high concentrations of chaotropic salts (Vogelstein and Gillespie, 1979).

In certain situations, even after increasing the stringency of PCR by titration of magnesium chloride and variation of annealing temperatures, multiple bands were present. In such cases, 20 µL of the PCR reaction and 3 µL of loading dye were loaded on a 2 % Nusieve (3:1 Agarose) gel in 1 X TAE (40mM Tris acetate, pH 8,

2mM EDTA buffer) and electrophoresis was conducted for 3 hr at 80 Volts to facilitate proper separation of the bands. The bands of interest were then excised with a sterile scalpel and placed in microtubes. DNA was extracted from the agarose slice using the protocols for Qiaex II gel extraction kit from Qiagen Inc. De Soto Avenue. Chatsworth, CA. In this procedure, instead of the traditional organic extraction with phenol-chloroform, the agarose gel containing the band of interest was incubated with a buffer (PB buffer) of high salt concentration and silica particles (QIAEX II) were added to the microtube. The reaction mixture was incubated at 50 ° C with frequent mixing every two minutes to keep the silica particles in solution. The principle of this procedure was to aid in solubilization of the agarose and allow the DNA to get adsorbed on the QIAEXII particles. Non-nucleic acid impurities such as agarose, proteins, salts and ethidium bromide were removed during the washing steps with another buffer that contained ethanol. In the final step, sterile water was used to elute the pure DNA that is now suitable for fluorescent sequencing and or labeling reactions in hybridization reactions.

- **Cycle Sequencing of the PCR products using ABI PRISM™ 310 Sequencer**

Cycle sequencing was conducted using The ABI PRISM ™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS according to the manufacturer's recommendations (Perkin Elmer Corporation, Foster City, CA). This technique is not only rapid and powerful, but is also simple as it is a single tube reaction that combines the unique properties of the enzyme for dye terminator sequencing and the convenience of the Ready Reaction Format. The Ready Reaction Format includes the dye-labeled dideoxynucleotides and the

deoxynucleotides at a concentration determined to produce a signal between base 10 and base 700+. PCR DNA (10-30 ng/μl), primer at a final concentration of 1 μM and 8 μl of the Terminator Reaction mix in a final volume of 20 μl adjusted with sterile water were cycle sequenced according to the following parameter: 95° C for 1min, 55°C for 10 sec, and 60 °C for 5 sec. These were repeated 25 times and ended with a final soak cycle at 4° C.

- **Spin Column Purification**

The samples were removed from the PCR machine and stored on ice until the columns were ready to use. The columns were hydrated for a minimum of 30 min at room temperature by adding 800 μl of water. Air bubbles were removed and the columns drained by gravity. To remove any interstitial fluid, centrifugation was conducted at 1300 x g for 2 min. The sample was carefully loaded onto the center of the column and centrifugation conducted as above. The purified sample was collected in a clean centrifuge tube and dried in a vacuum centrifuge without heat.

- **Preparing and Loading samples**

The samples were removed from the vacuum centrifuge and resuspended in 25 μl of template suppression reagent (P/N 401674 from Perkin Elmer). The samples were mixed thoroughly by vortexing followed by brief centrifugation. The samples were denatured at 95° C for 2 min and mixed again by vortexing followed by brief centrifugation and then transferred to special 0.2 ml capillary tubes to be loaded on the ABI PRISM™ 310 sequencer.

3.1.2 Construction of the cDNA sequence

3.1.2.1 RNA Extraction

All instruments and materials needed for excision of liver and extraction of RNA were sterilized with water treated with diethylpyrocarbonate (DEPC) to remove any RNAses. Total RNA was isolated from excised livers of *Gambusia affinis* using TRI-REAGENT (Molecular Research Center™ Inc.) that contained phenol and guanidine thiocynate followed by a series of centrifugation steps. RNA was then precipitated with 3.5 M sodium acetate and chilled isopropanol. Pelleted RNA was dissolved in diethylpyrocarbonate (DEPC) treated water. Purity and amount of RNA were determined using a Beckman DU-600 spectrophotometer at 260 and 280 nm.

3.1.2.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The cDNA sequence of the *Gambusia affinis* p53 gene was isolated *via* the reverse transcriptase-polymerase chain reaction (RT-PCR) using a gene-specific 3' primer. About 0.2 μg RNA in about 32 μl of DEPC-treated water was added to 3 μl (2.5μM) of 3' primer and incubated at 70° C in a incubator. The reaction temperature was reduced to 45 °C after which about 1 μl of Maloney Murine Leukemia Virus reverse transcriptase (MMLV-RTase; Stratagene, LaJolla, CA) (20 U/μl), 5μl DTTs (0.1M), 2μl dNTPs (25mM) and 5μl of first strand buffer was added and the mixture was mixed gently and incubated at 37° C for 1 hr. The completed reaction was immediately placed on ice.

For the gene-specific RT-PCR, a portion of the RT using the 3' gene-specific anchor primer was amplified using standard polymerase chain reaction. (PCR)

conditions (2.0mM $MgCl_2$), 2 μM dNTPs, 10mM Tris.HCl (pH 8.3), 50 mM KCl. To this reaction 0.5 μM 5' gene specific primer, 0.5 μM 3' primer and 1U Taq polymerase™ were added. Amplification reactions were set for 30 cycles with 95° C for 1 min, 70 ° C for 1 min and 72° C for 2 min. A final incubation for 7 min was included for extension of unfinished products.

3.1.2.3 Gel Electrophoresis and Detection

RT-PCR products were analyzed by electrophoresis on 1.5% agarose-1X TAE (40mM Tris acetate, pH 8, 2mM EDTA) against suitable molecular weight markers. Products were visualized by ethidium bromide staining and transillumination at 302-nm UV light.

Direct DNA sequencing of purified RT-PCR-DNA was conducted as mentioned as above.

3.2 Southern Hybridization and Detection

According to the Current Protocols (Sambrook et al., 1989), the principle of hybridization can be defined as a process in which a single–stranded DNA or RNA molecule of defined sequence (the "probe") can base-pair to a second DNA or RNA molecule that contains a complementary sequence (the "target"). The probe is labeled either by using fluorescent, biotin or radiolabeled techniques, while the target is immobilized on a membrane support. The stability of the hybrid will depend upon the extent of the base pairing between the target and the probe. This technique has widespread applications in the identification of single-copy genes in complex genomes and

the sensitivity of the technique is highly dependent on the labeling procedure, purity of the probe and concentration of the probe and target DNA.

Labeling DNA probes with non-radioactive procedures (e.g. fluorescent, biotin) is increasing becoming popular because they are safe to handle, have no disposal problems associated with radioactive isotopes and generally have long shelf life (1 yr.). Labeling with digoxigenin (DIG), is basically a non-radioactive procedure in which a steroid hapten couples to dUTP *via* an alkali-labile ester-bond. It is a random primed process based on the hybridization of random oligonucleotides to the denatured DNA template. The Klenow enzyme uses the 3'-OH termini of the oligonucleotides and a mixture of deoxyribonucleosides containing DIG-11-dUTP to synthesize the complementary strand of DNA. This results in the incorporation of the digoxigenin into the newly synthesized DNA.

According to the protocol provided by the manufacturer (Boehringer-Mannheim Biochemicals), the standard reaction with 1 μg control DNA yields about 250 ng of DIG-labeled DNA. Labeling efficiency is directly correlated to the size (700 base pairs), amount and purity of the of the DNA fragments. DNA fragments smaller than 200 base pairs and larger than 2000 base pairs are not efficiently labeled. The same principle holds for the purity of the template. The template should be free of primers, unincorporated deoxynucleotides and enzymes. In addition, about 1 μg of DNA is needed for efficient labeling. The hybridized probes are then immunodetected with antidigoxigenin-alkaline phosphatase. The fragments are then visualized with chemiluminiscence substrate Lumigen PPD.

Southern hybridization and detection basically involve 4 steps: Southern Blotting, labeling, hybridization, and chemiluminiscent Detection.

3.2.1 Southern Blotting

3.2.1.1 Restriction Digest and Gel Electrophoresis

This is usually the first stage of a hybridization experiment. *Gambusia affinis* genomic DNA (target) at a concentration (5.4 μg/μl) was incubated with EcoRI, BamHI and EcoRI and Bam HI (New England Biolabs) at 37°, 30 °C and 37 ° respectively for 4 hours. Twenty micro litres of the restricted DNA was loaded on a 1% Nusieve (3:1 Agarose, FMC Bioproducts) gel in 1X TAE buffer (Amresco) and electrophoresis was conducted for 4 hr at 60 volts. The DNA fragments were visualized by staining with ethidium bromide. The gel was submerged in 0.25 N HCl and shaken gently at room temperature for 10 min and rinsed in sterile water briefly. The gel was then submerged in denaturing and neutralization solutions for 45 min each time with constant but gentle agitation.

3.2.1.2 Southern Transfer

For the process of Southern transfer of DNA, appropriate sizes of positively-charged nylon membranes are prepared according to the manufacturer's recommendations (Boehringer Mannheim). The nylon membranes and the filter papers were equilibrated in 2x SSC and 10x SSC respectively. DNA was transferred from the gel to the nylon membrane overnight by capillary action (Figure 2.4) A washing step with 5x SSC was also included to remove any debris and contaminants that could be introduced during the transfer process. DNA was then permanently fixed to the membrane by baking in an oven at +80°C for 1 hour.

3.2.2 DNA Labeling with Digoxigenin (DIG)

- **Reagents and Materials**

All reagents were purchased from Boehringer Mannheim Biochemicals unless otherwise mentioned. A Genius 1 DNA labeling and detection kit™ contained the following components:

10 X Hexanucleotide mixture, 10 X DIG DNA labeling mixture, Klenow enzyme, labeling grade, 2 Units/µl, DNA dilution buffer, Labeled control DNA 5 ng/µl,

Anti-dig-alkaline Phosphatase, Blocking reagent, Glycogen solution (20 mg/ml), nylon membrane (positively charged)

- **Additional Reagents and Materials**

200 mM EDTA, pH 8.0, 4 M lithium chloride, 100% ethanol, 70% ethanol, TE/SDS buffer, sterile water, Genius Buffer I: 100 mM Tris-HCl, pH 7.5 and 150 mM NaCl Genius Buffer II: 2 g Blocking Reagent and 100 ml Genius Buffer I. Substrate Mixture: Nitroblue tetrazolium salt (NBT), X-phosphate solution and Genius Buffer III Tris-Ethylenediaminetetraacetic acid (EDTA) (TE) with sodium dodecyl sulfate (SDS): 1x TE (10 mM Tris-HCl, 1mM EDTA) 0.1% SDS, pH was adjusted to 7-8

DNA dilution buffer: 50 µg/ml herring sperm DNA, 10 mM Tris-HCl and 1mM EDTA

For Genius Buffer II, the above reagents were added and heated to 60° C. The volume was made to 100 ml with sterile water and aliquots were stored at –20 ° C.

Genius buffer III: 100mM Tris-HCl, pH 7.5, 100 mM NaCl (Adjust pH to 9.5)

50 mM $MgCl_2$.

For Genius buffer III, 5.844 g of NaCl and 100 ml of 1 M Tris-HCl pH 7.5 were added to 700 ml of sterile bottled water and pH adjusted to 9.5 with 14-16 ml of 10 N NaOH. The volume was made up to 1000 ml with 1M $MgCl_2$

3.2.2.1 Purification of DNA Fragment

A PCR product of 450 base pairs from *Gambusia affinis* was generated according to the protocol in section 3.1.1.2. PCR was optimized to obtain a single band. Another PCR product of 350 base pairs was generated from rainbow trout corresponding to exons 7-8. The DNA fragments were purified of unincorporated nucleotides, primer dimers, and enzymes using Micro-con 100 columns (Amicon, Inc). The PCR products were mixed with 1 x Tris EDTA buffer to final volume of 500 µl and added to columns. The mixtures were centrifuged at 2000 rpm for 15 min. The filters were inverted and placed in a sterile microcentrifuge tube and centrifuged again at 4000 rpm for 5 min to elute DNA. The concentration of the purified fragments were determined using a Beckman DU-600 spectrophotometer at 260 and 280 nm.

3.2.2.2 Scale Up Random Primed DNA Labeling

The purified DNA was labeled using the Scale-up Random primed method with digoxigenein-11dUTP (DIG) according to the protocols furnished by Boehringer-Mannheim Biochemicals. The protocols detailed here were initially performed for exons 5-6 and then repeated for exons 7-8.

Reagent mixture and purified DNA were prepared by adding the following reagents in a total volume of 100 µl with sterile water:

Reagent mixture: 10 x Hexanucleotide mix 10 µl

10 x Dig DNA labeling mix 10 µl

Klenow enzyme 2 Units/µl 5 µl

Purified DNA 1µg/10µl

Sterile bottled water 65 µl

The purified DNA was denatured for 10 min in a boiling water bath and quickly chilled on ice for 30 sec. Of the total volume 25 µl of the reagent mixture was added and incubated overnight in a 37° C water bath. The reaction was terminated by adding 10 µl of 200 mM EDTA to the DIG-labeled DNA and 1 µl Glycogen (20 mg/ml). The DIG-labeled DNA was precipitated by adding 11.1 µl (0.1 volume) of 4 M lithium chloride and 311.3 µl of chilled 100% ethanol. The reaction mixture was mixed thoroughly and incubated at –70 °C for 30 min. After 30 min, it was thawed and centrifuged at 11,000 rpm for 20 min. DNA formed a pellet and was washed with 1 ml of chilled 70% ethanol to remove any traces of the salt. The supernatant was decanted and the pellet dried and suspended in 50 µl of TE/SDS buffer with a 10 min incubation at 37 °C. The concentration of the DIG-labeled DNA and the DIG-labeled control DNA was estimated according to a colorimetric method and is described below. The DIG-labeled probes were serially diluted to determine their concentration and are explained in Table 3.3.

The DIG-labeled DNA was serially diluted as described in the Table 3.3. On a positively charged nylon membrane, 1µl of each dilution labeled DNA was spotted and the membrane baked in an oven at +80 °C for 30 min.

Table 3.3 Estimation of the concentration of DIG-labeled control and DIG-labeled probe

DIG Labeled Control	DIG Labeled Probe	Dilution Factor	Concentration
A	1	1:5 2µl/8µl DNA Dilution Buffer	1 ng/µl
B	2	1:50 2µl/18µl DNA Dilution Buffer	100 pg/µl
C	3	1:500 2µl/18µl DNA Dilution Buffer	10 pg/µl
D	4	1:5000 2µl/18µl DNA Dilution Buffer	1 pg/µl
E	5	1:50000 2µl/18µl DNA Dilution Buffer	0.1 pg/µl

- **Colorimetric Detection of the Concentration of the DIG-labeled Probes**

The membrane was placed in a petri dish containing Genius Buffer I. The buffer was decanted and replaced with Genius Buffer II and incubated at room temperature for 5 min. Anti-dig alkaline phosphate/Genius buffer II at a 1:5000 was added and the membrane incubated at room temperature for an additional 5 min. The membrane was subsequently washed twice in Genius Buffer I at room temperature with 5 min for each wash. Finally the membrane was incubated in Genius buffer III at room temperature for 2 min.

A substrate mixture was prepared with the following reagents in the dark as the reagents are light sensitive. 10 ml of Genius Buffer III, 45 µl of NBT, and 35 µl of

X-phosphate solution. For color development the substrate mixture was added to the membrane and incubated at room temperature for 30-60 min with constant but gentle agitation. The optimum concentration of the DIG-labeled DNA was determined by comparing the color development of the serially DIG-labeled DNA with that of the serial dilutions of DIG-labeled control DNA. This was repeated a second time for the other DIG-labeled probe.

3.2.3 Hybridization

- **Materials**

Reagent Mix 1 : 5 X SSC . 0.1% N-lauroylsarcosin. 0.02% Sodium dodecyl sulfate (SDS)

Reagent Mix 2: 4% Blocking reagent (Catalog # 1096176) and 200 μg/ml Herring sperm (Boehringer Mannheim Biochemicals).

Formamide. 2 X Washing buffer: 2 X SSC in 0.1% Sodium dodecyl sulfate.

5x washing buffer: 0.5 x SSC in 0.1% Sodium dodecyl sulfate. hybridizing screw-cap bottle, DIG labeled DNA probe at 10 ng/ml and hybridization oven.

Reagent Mix 1 was initially prepared by adding the reagents and heating to 60 °C with constant stirring. Blocking reagent and herring sperm (200 μg/ml) were added after all the reagents had dissolved completely.

3.2.3.1 Prehybridization

To decrease the intensity of the background signal, the non-specific nucleic acid binding sites on the membrane need to be blocked. Hybridizing the membrane in prehybridization solution for at least 2-3 hr aided in blocking the sites.

- **Method**

An equal volume of formamide and modified prehybridization solution were added and used as a working modified prehybridization solution. The nylon membrane that was used for Southern transfer was placed in a hybridizing screw-cap bottle containing 14 ml of working modified prehybridization solution and pre-hybridized at 50 ° C for at least 2.5 hr. The DIG-labeled probe was diluted to a concentration of 10 ng/ml in the working modified prehybridization solution.

The hybridization solution was denatured in a boiling water bath for 10 min prior to use. The working modified prehybridization solution was replaced with the hybridization solution and hybridization was conducted at 50 °C overnight in a hybridization oven. The membrane was washed in 2 X washing buffer twice at room temperature for 5 min each time. In addition, as the probes were more than 100 base pairs the membrane was washed in 0.5 X washing buffer twice at 65 °C for 15 min to increase the stringency of hybridization.

- **Chemiluminescent Detection**

Genius Buffer I: 100 mM Tris-HCl, pH 7.5, 150 mM NaCl; Genius Buffer II: 2% Blocking Reagent/ Genius Buffer I; Genius Buffer III: 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, Adjust pH to 9.5 with 7-8 ml of 10 N NaOH and 50 mM $MgCl_2$; 0..2% Tween 20/Genius buffer I

Anti-DIG-Alkaline Phosphatase/Genius buffer II at 1:5000

Lumigen PPD 10 mg/ml Catalogue # 1357328, Boehringer Mannheim Biochemicals) Biomax Light-1Xray film (8x 10 in) Cat # 1788207, Kodak

Sheet protector (Clear plastic) 8 ½ x 11 inches

- **Methods**

The chemiluminiscent detection method basically consists of 3 steps:

Step1: The membrane is treated with Blocking reagent to prevent non-specific attraction of the antibody to the membrane. The membrane was equilibrated in 15 ml of 0.3% Tween 20/Genius Buffer I for 1min after the post hybridization washes and then blocked in 15 ml of Genius Buffer II for ~ 60 min.

Step2: The membrane was then incubated in 15 ml of Anti-DIG-Alkaline Phosphatase/Genius buffer II at 1: 5000 for 30 min. Care was taken to transfer the membrane to a clean tray and was washed in Genius buffer I twice at room temperature, 15 min for each wash.

Step3: The membrane carrying the hybridized probe and bound antibody is reacted with Lumigen PPD and exposed to X-ray film to record chemiluminiscent detection.

The membrane was then equilibrated in Genius Buffer III for 2 min after which the membrane was placed between two pieces of clear plastic and 500 µl of substrate solution was distributed evenly over the surface of the membrane taking extreme care to keep the substrate solution away from light. Excess substrate solution was removed and the membrane exposed to a X-ray film in a dark room at room temperature for 30 min-1 hr. The X-ray film was developed to view the results.

3.3 Screening for Mutational Events

In previous experiments in our laboratory, Law et al., (1994) exposed *Gambusia affinis* to methylazoxymethanol acetate (MAM-Ac; Sigma Chemical company,

St. Louis, MO). The specimens were removed to grow-out tanks and sampled periodically. Tumor growth was observed in 26 weeks. The fish were anesthetized with tricaine methane sulphonate (MS-222), examined grossly and fixed in buffered zinc formalin (Z-FIX, Anatech, LTD., Battle Creek, MI) for 15-17 hr. The specimens were embedded in paraffin and used for studying changes in cell proliferation, apoptosis and changes in p53 expression. Dr. Law (Assistant Professor, North Carolina University) kindly provided sections used in the study and the details of the specimens are included in Table 3.4. DNA was isolated from the paraffin embedded tissues according to the protocol provided by Dr. Dave Malarkey (personal communications, Dr. Malarkey, Laboratory of Molecular Toxicology, NIEHS). PCR and direct sequencing of the PCR products are described below in sections 3.3.1-3.3.3.

3.3.1 DNA Isolation from Paraffin Embedded Tissues

Excess paraffin was removed by scraping with a sterile scalpel blade. Very thin sections (<10 μM) were obtained with a microtome and placed in a sterile 1.5ml centrifuge tube. A brief centrifugation was conducted to collect the tissue at the bot - tom. Xylene extraction was conducted 2 times, followed by two ethanol washes (100% and 95% respectively). The microcentrifuge containing the tissue was placed in a speed vacuum for 20 min. DNA extraction was conducted with 200 μl of digestion buffer that contained 50mM Tris, 1mM EDTA, 0.5% Tween 20 and 0.2 mg/ml proteinase K. The samples were incubated overnight in a 55° C water bath. A mixture

Table 3.4 Diagnosis comments of *Gambusia affinis* exposed to MAM-Ac

Specimen number	Sex of animal	Diagnosis Comments
PD90EA470	Male	Liver Carcinoma (hepatocellular exocrine pancreatic); extensive cytotox with pseudo lobulation ; large eosinophilic cytoplasmic droplets
PD90EA471	Female	Liver Carcinoma(hepatocellular exocrine pancreatic?) extensive cytotox; numerous eosinophilic granules
PD90EA473	Male	Liver Carcinoma
PD90EA476	Female	Liver Adenoma
PD90EA486	Female	Liver Carcinoma

of phenol:chloroform:isoamyl alcohol (25:24:1) was added. DNA was precipitated with 1/10 volume of 1.5 M sodium chloride and 400 µl of 100% ethanol. DNA formed a pellet after a brief centrifugation and was washed with 70% ethanol to remove any traces of salts. The pellet was dissolved in 1X Tris EDTA buffer and was subsequently dried in a speed vacuum for 20 min and then resuspended in 50 µl of sterile distilled water. For amplification, 2 µl of the suspension was used in a PCR.

3.3.2 PCR Amplification

PCR was set up as discussed in 3.1.1.2. Forward primers was selected from exons 5 and reverse primer from exon 6 (C1 and GR1 and NH1 and NH2R respectively, Table 3.1).

3.3.3 Sequencing of PCR products

Cycle sequencing was conducted according to the protocol described in section 3.1.1.5.

Chapter 4

Results and Discussions

This chapter describes the results obtained using the methodology detailed in Chapter 3. Although techniques such as PCR, sequencing, and southern hybridization have been studied and reported by numerous investigators in the field of molecular biology for mammalian species, protocols for conducting the same are few for fish species (Soussi et al., 1992; Krause et al., 1997). As such, each of the individual process had to be optimized and standardized to suit our condition.

4.1 p53 Gene Sequence of Exons 5-8 in *Gambusia affinis*

Livers excised from five adult *Gambusia affinis* weighed ~110 mg on an average. Protocols for genomic DNA extraction were based on Current Protocols (Sambrook et al., 1989) with certain modifications. While the concentration of genomic DNA was determined to range from 3.6-5.5 μg/μl, the concentration of RNA was 5 μg/μl. Purity of the genomic DNA and total RNA were determined by obtaining the ratio of absorbance of nucleic acid at 260 nm and 280 nm (A_{260}/A_{280}). It was found to range between 1.82 – 1.9 and 1.9-2.0 for genomic DNA and total RNA, respectively. Rainbow trout DNA concentration was 7.69 μg/μl and the A_{260}/A_{280} ratio of DNA absorbance was 1.85.

According to Current Protocols (Sambrook et al., 1989) the A_{260}/A_{280} ratio for DNA and RNA should be 1.8 and 2.0 respectively. If the A_{260}/A_{280} ratio is below 1.7, it indicates that the nucleic acid preparation is not pure and that the contamination

could be due to UV-absorbing materials. Many molecular biology scientists have speculated on the validity of the ratio of absorbance of nucleic acids (Glassel, 1995). According to Glassel (1995) the two–wavelength method of Warburg and Christian (1942) is being used indiscriminately. Despite the fact that the two-wavelength method was initially developed to determine the degree of contamination of proteins by nucleic acids, it is being used the other way to determine the degree of contamination of nucleic acids by proteins. Glassel (1995) further criticizes the concept in that the specific absorption coefficients of nucleic acids being greater than proteins, the contribution of minor amounts of proteins to the ratio is negligible. However, other investigators such as Manchester, (1995) recommend not to stop measuring the ratio, but to interpret the ratio with extreme caution. Concentration of genomic DNA of *Gambusia affinis* was also determined by conducting electrophoresis of an aliquot of DNA and RNA at 80 volts for 2hr. The DNA was then visualized by ethidium bromide staining. DNA appeared as a smear with the high molecular weight DNA on the top part of the agarose gel.

4.1.1 DNA - PCR

PCR is an indispensable technique in most molecular biology laboratories. Among the various variables that need to be optimized for PCR, the most important ones are the concentration of magnesium, annealing temperature, buffer pH, and cycling conditions. PCR can work like a double-edged sword when solely employed to generate large amounts of a desired product. Failure to amplify a sample under optimum conditions can result in the generation of spurious bands and exclusion of the desired product. The situation is complicated due to the interdependence of some of

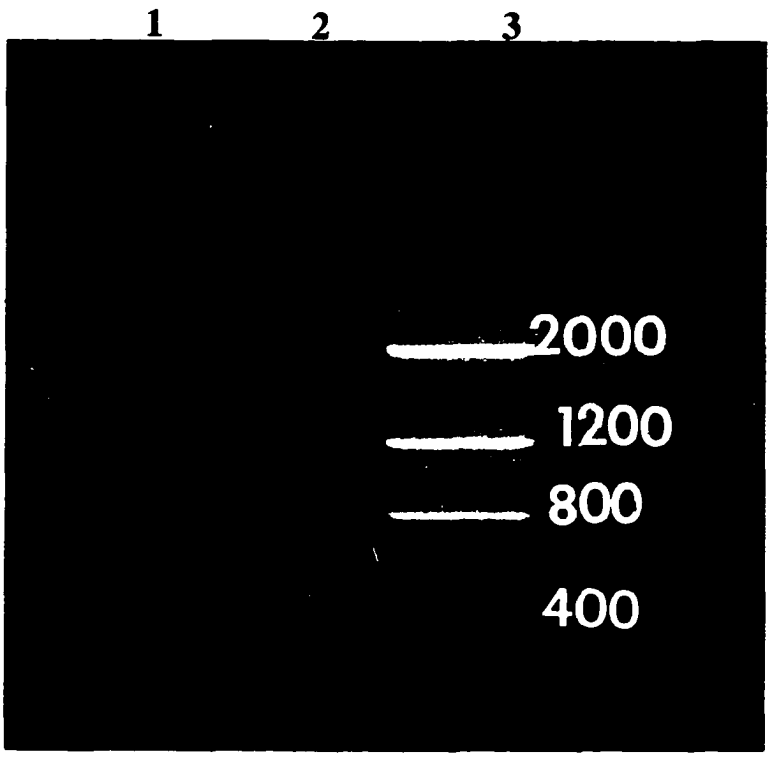
the variables. An example is dNTPs and Mg^{2+}. In solution dNTPs can chelate Mg^{2+} ions. Increasing the concentration of the dNTPs without optimization can result in the decrease in the availability of the free Mg^{2+} concentration leading to no amplification.

Mg^{2+}concentration was titrated at 0.5 mM increments ranging from 1.5-4 mM. While the presence of excess Mg^{2+} can result in spurious bands, concentration lesser than the optimum can result in no amplification. The Mg^{2+} concentration was optimized at 1.75 mM for exons 5-6. The primers were selected from the p53 gene sequence of rainbow trout for the regions 5-8 (Krause et al., 1997). The melting temperature (Tm) of the primers (NH1 and NH2R, Table 3.1) was obtained by the Oligo 5.0™ program and also by calculations based on the following formula:

Tm = (G + C)*4 + (A+T)*2, Where A and T are the purines; adenine and guanine and C and T are the pyrimidines; cytosine and thymine respectively.

An applicable annealing temperature is 5 degrees below the Tm of the primers. However, the Tm obtained by using computer programs such as Oligo 5.0™ or by calculations based on the number of purines and pyrimidines is always going to be an approximation as the oligonucleotide sequences are based on the sequence of rainbow trout p53 gene and not that of mosquito fish. A single difference in a base can result in the change of the Tm by as much as 5 degrees. This is more often the case when identification and isolation of genes is initiated across species barriers. That is a gene sequence in a new species is initiated based on the sequence information of the same gene in a related species (Roux, 1994). The positive control with rainbow trout DNA produced a PCR product that was similar in size to the *Gambusia affinis*. Negative controls did not show any amplification that indicates that false positives were

avoided. The reagents were UV irradiated by placing tubes containing the reagents on a UV light box for 5 min. (Cone et al., 1990). The principle behind UV irradiation of reagents to prevent PCR contamination is based on the formation of cyclobutane rings between neighboring pyrimidines. The cyclobutane rings form intra-strand pyrimidine dimers that inhibit the elongation of bases initiated by the DNA polymerase (Cone et al.,1990). The melting temperature of the NH1 (66 °C) and NH2R (70 °C) primers were relatively high. The annealing temperature was varied every 2 °C after selecting 65 °C as a starting point. A 450 base pair product was observed at 60 °C annealing temperature (Figure 4.1). DNA size marker (Low DNA mass ladder, Life Technologies, Gibco-BRL,Gaithersburg, MD) was loaded in every gel to estimate the size of the bands. The quality of the PCR reaction is frequently compromised due to artifacts such as primer-dimers and non-specific priming which are detected as lower molecular weight bands on agarose gels. Primer dimers and non-specific priming frequently occur in PCR because Taq DNA polymerase is active over a broad range of temperature conditions and primer annealing and extension occur even with brief incubations of the reaction mixtures well below the annealing temperatures of the primers (Innis et al., 1990). These PCR artifacts were eliminated to a large extent with the Hotstart technique (Perkin Elmer Applied Biosystems, Foster City, CA) described in section 3.1.1.2. Nucleotide sequencing of the PCR products (NH1-NH2R) using the ABIPRISM™310 automated sequencer resulted in obtaining a 450 base pair sequence (Figure 4.2a). The sequence information was confirmed using both the forward and reverse primers. The instrument control pGEM was also included during sequencing



Agarose gel electrophoresis of PCR product (NH1-NH2R) 450 base pairs spanning exons 5-6 and intron in between of *Gambusia affinis* p53 gene. Lane 1: Rainbow trout DNA, Lane 2: *Gambusia affinis* DNA. and Lane 3: Low DNA Mass Ladder

Figure 4.1 PCR detection of the p53 gene spanning exons 5-6 of *Gambusia affinis*

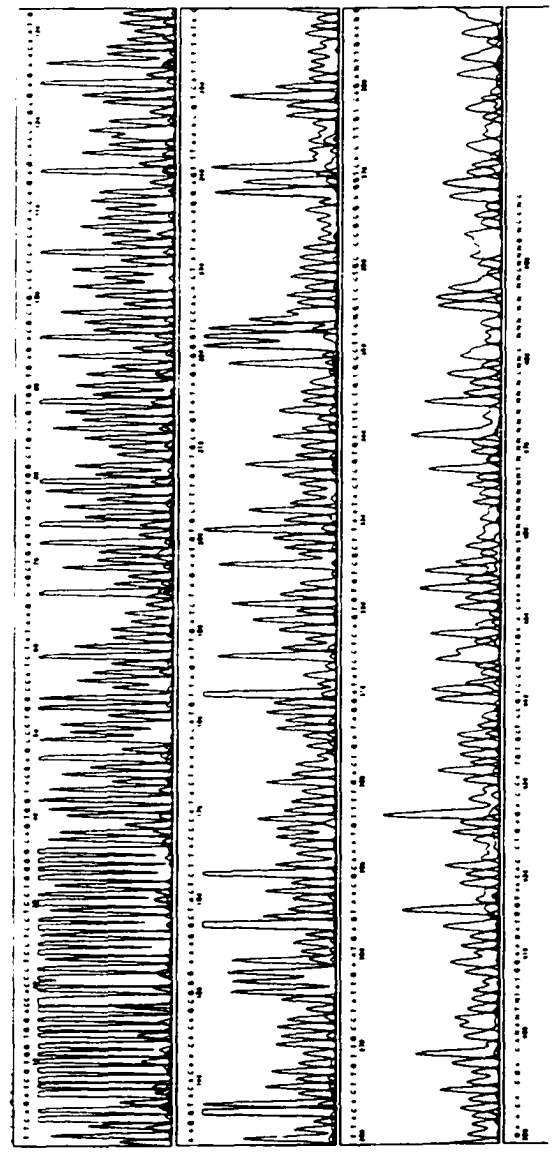


Figure 4.2a DNA sequence of exon 5, intron, and exon 6

and its sequence information was confirmed with the information provided by Perkin Elmer. The sequence confirmed well which indicates that the sequencer was working according to the specifications of the manufacturer (Figure 4.2b). The sequence of NH1-NH2R of *Gambusia affinis* was then aligned with human and rainbow trout (*Onchorhynchus mykiss*) sequences from that region (Figure 4.3). The alignment of the exon 5-6 sequence from *Gambusia affinis* with that of human and rainbow trout infers that there is a high homology between the species (Figure 4.3). In the human sequence, the exon 5-6 is also a region where most of the mutations are observed. Within exons 5-6 two extra sets of primers were selected. These primer combinations (C1-GR1 and C1-I5R) (Table 3.1, Figure 4.4a and 4.4b) have the same forward primer (C1) in exon 5. While the reverse primer in the former primer combination (GR1) was located in exon 5, the reverse primer in the second set was located in the intron in between exon 5 and 6 (I5R). The former primer combination (C1- GR1) resulted in a PCR product of 160 base pairs and the latter (C1-I5R) resulted in a PCR product of ~200 base pairs. The sequence from the C1-GR1 PCR product of *Gambusia affinis* was aligned with sequence information from rainbow trout from the same region (Figure 4.5). The sequences aligned well indicating high homology in the region.

Cloning of PCR products into pCR2.1™ and/or pCR II™ vectors from Invitrogen (Carlsbad, CA) was performed as described in section 3.1.1.4. The clones were labeled JMH1-1 and JMH1-6. The orientation of the clones is depicted in Figure 4.6. The concentration of the clones was ~ 225 ng/μl and the total volume of suspension

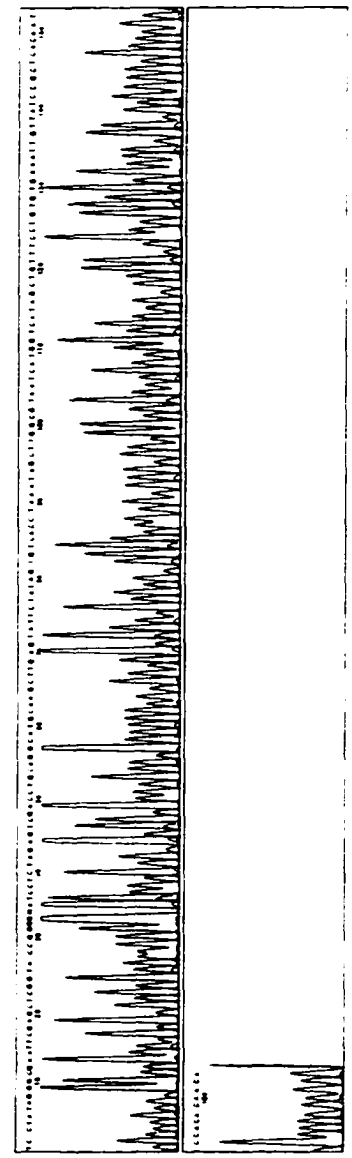
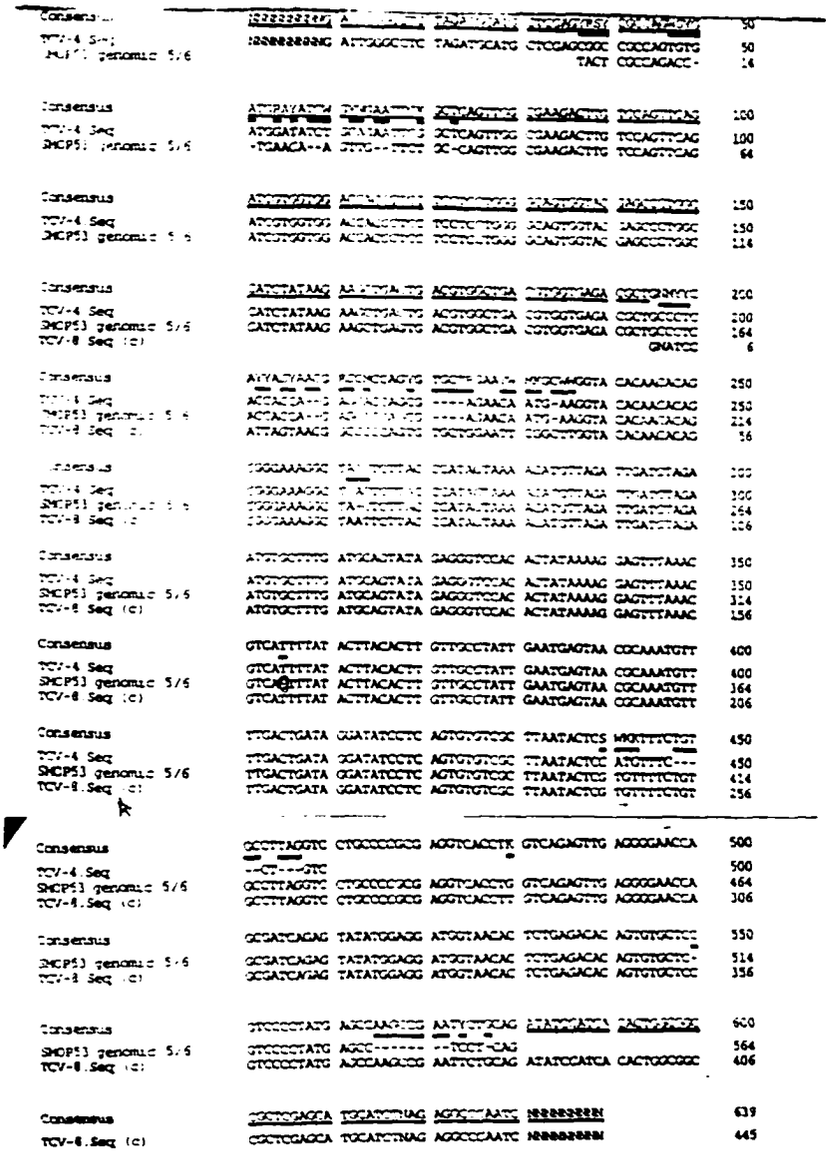
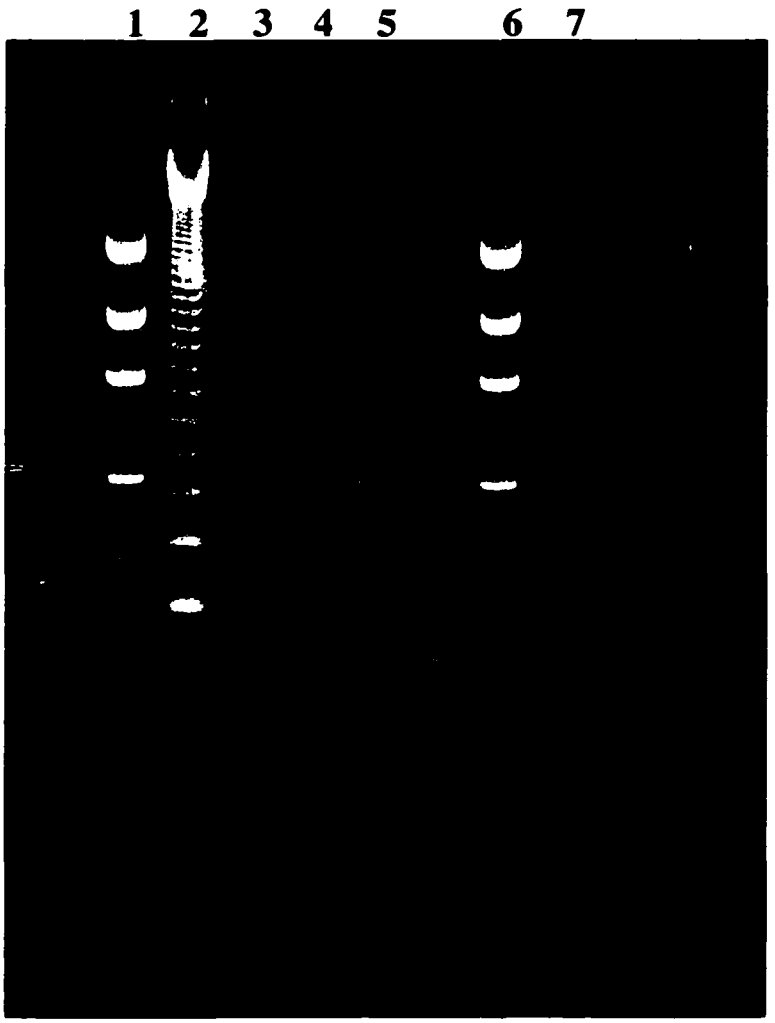


Figure 4.2b Sequence of pGEM ABIPRISM™ sequencer control



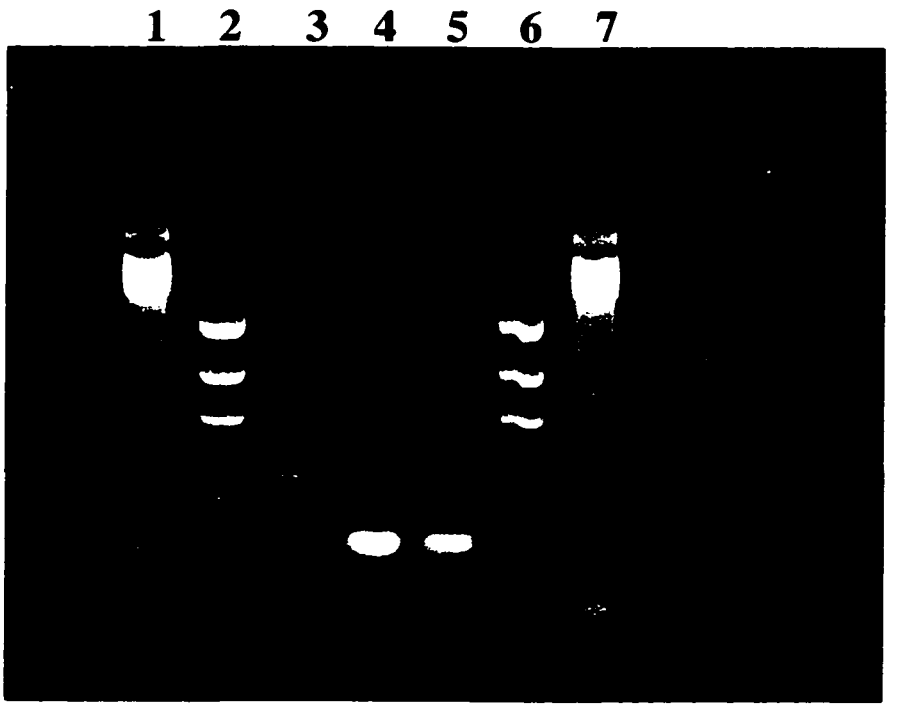
TCV-4 and TCV-8 correspond to *Gambusia affinis* clones
SMop53 corresponds to rainbow trout clones

Figure 4.3 Alignment of exons 5-I-6 sequences of p53 gene in *Onchorynkus mykiss* (Rainbow Trout) and *Gambusia affinis*



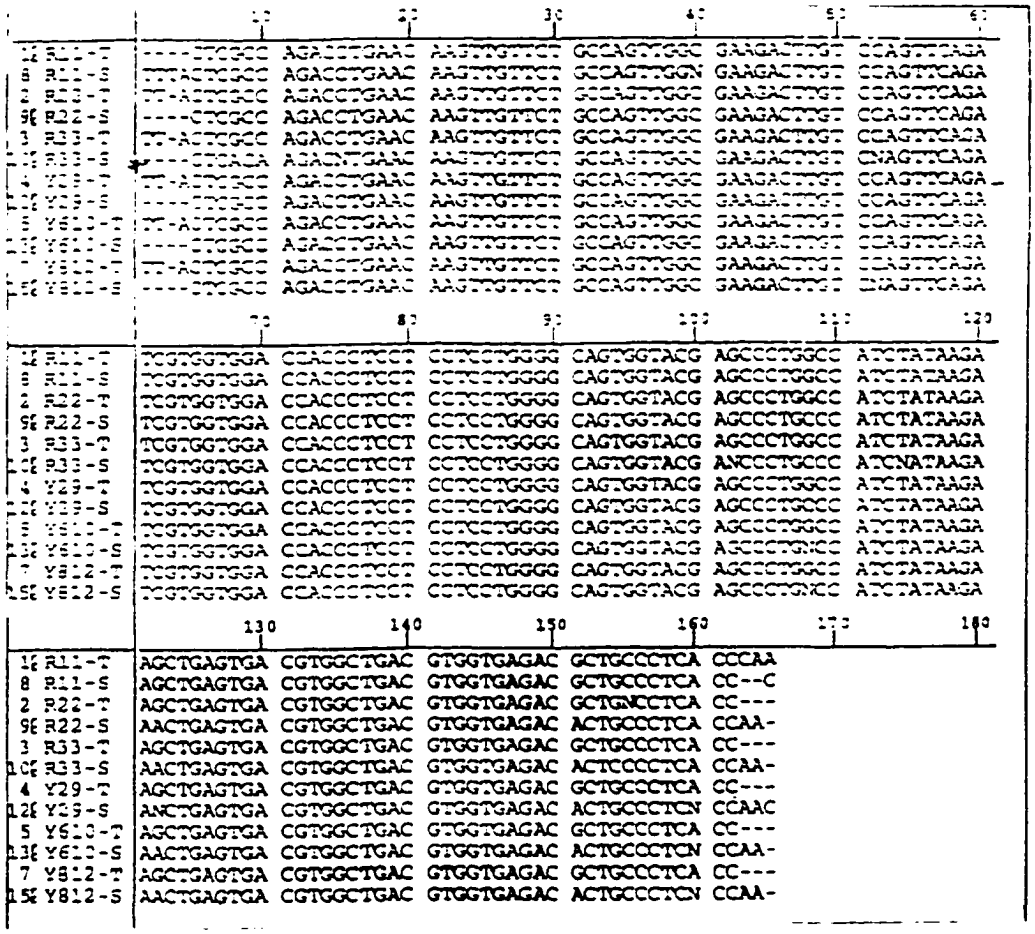
Agarose gel electrophoresis of PCR products (C1-GR1) of 160 base pairs. Lanes 1and 7: Low DNA Mass Ladder, Lanes 2: 123 base pair Marker, Lanes 3 and 4: 160 base pairs of *Gambusia affinis* DNA, and Lane 5: 160 base pairs of Rainbow trout DNA.

Figure 4.4a PCR detection of p53 gene in *Gambusia affinis* with internal primers from exons 5-6



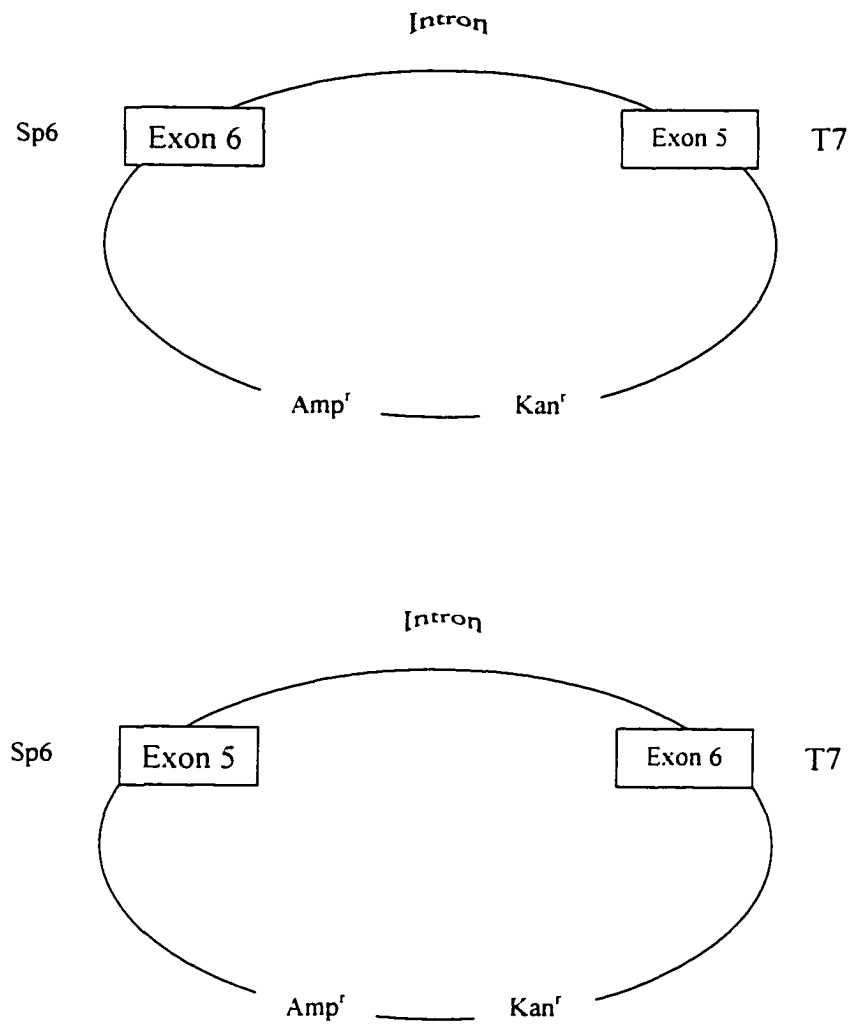
Agarose gel electrophoresis of PCR products (C1-I5R) of 250 base pairs spanning Exon 5 and intron in between. Lane 1 and 7: 123 base pair Marker, Lanes 2 and 6: Low DNA Mass Ladder, Lane 3: 450 base pairs (NH1-NH2R), Lane 4 and 5: 250 base pairs of *Gambusia affinis* DNA.

Figure 4.4b PCR detection of the p53 gene in *Gambusia affinis* spanning exon 5 and intron 5



R11-T, R11-S, R22-S, R22-T,R33 –S, and R33-T correspond to rainbow trout clones; Y29-S , Y 61—T and Y812-S correspond to clones of *Gambusia affinis*

Figure 4.5 Alignment of *Gambusia affinis* and Rainbow Trout p53 genes in regions spanning exons 5-6



Cloning of the PCR products (JMH1-1 and JMH1-6) into pCR2.1™ (Invitrogen, Inc.,)

Figure 4.6 Orientation of the cloned PCR products of exons 5-6 of *Gambusia affinis*

was 50 µl for each clone. Therefore, the total amount of each of the cloned product was 225 ng/µl * 50µl = 11250 ng or 11.25 µg. The cloned PCR products were sequenced in both the directions to confirm the sequence of the insert. The sequence information obtained using the reverse primer is included as Figure 4.7. On either side of the product-insert restriction sites for EcoRI are present. The cloned PCR product obtained using the primers NH1 and NH2R was then restricted with EcoRI to obtain the insert (Figure 4.8).

Sequencing the PCR products directly as well as by cloning followed by sequencing resulted in obtaining the sequence corresponding to exon 5-6 of *Gambusia affinis* p53 gene. The sequences compared well with the earlier sequences obtained using NH1 and NH2R as primers. Amplification of the regions encompassing exon 5-6 by selecting nested primers from the intron regions unequivocally confirms the sequence of the p53 gene for this region.

In order to obtain the sequence of exons 7-8, two sets of primers combinations (NHF7-NHBR1 and NH3-NHBR1) (Table 3.1) were selected for PCR and sequencing. A PCR product of ~ 400 base pairs was generated with NHF7-NHBR1 (Figure 4.9). However, amplification in the region corresponding to exon 7-8 was difficult and various primer combinations had to be tried initially. The OptiHotStar™ from Invitrogen was used to amplify genomic DNA for exons 7-8. The protocol to use the OptiHotStart™ is explained in detail in section 3.1.1.2 (Table 3.2). Of the four different PCR buffers and three different Mg^{2+} concentrations, amplification was successful only for

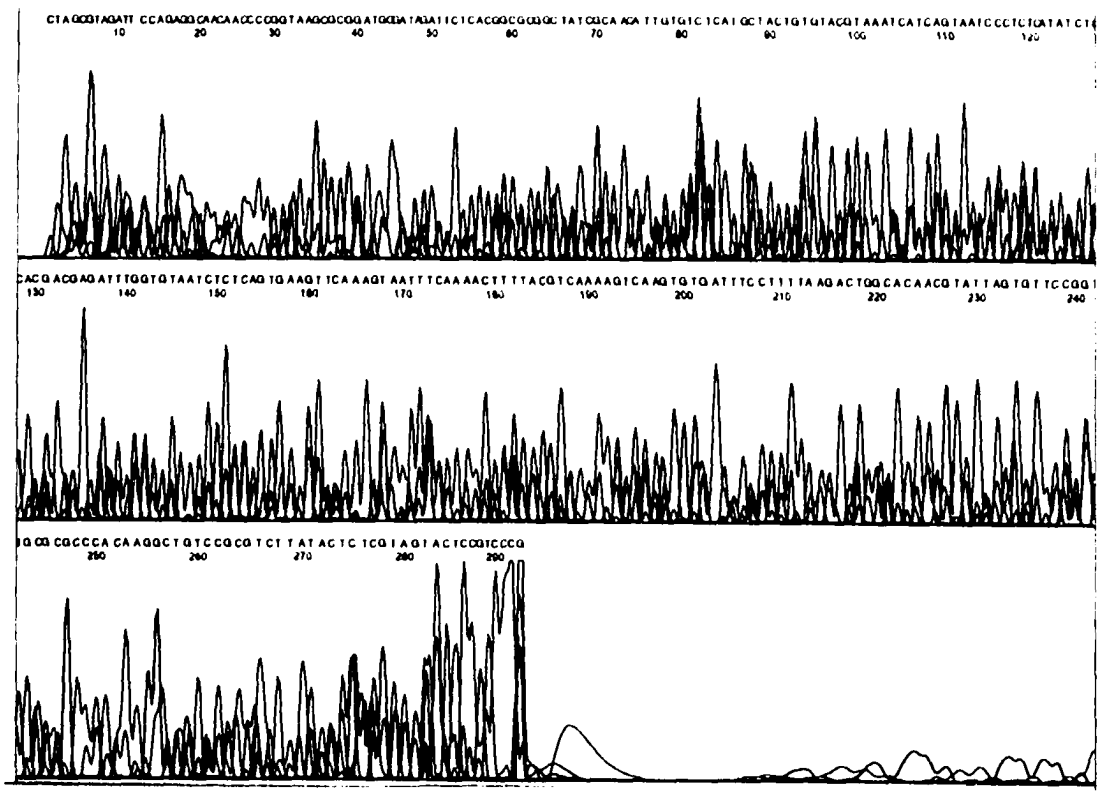
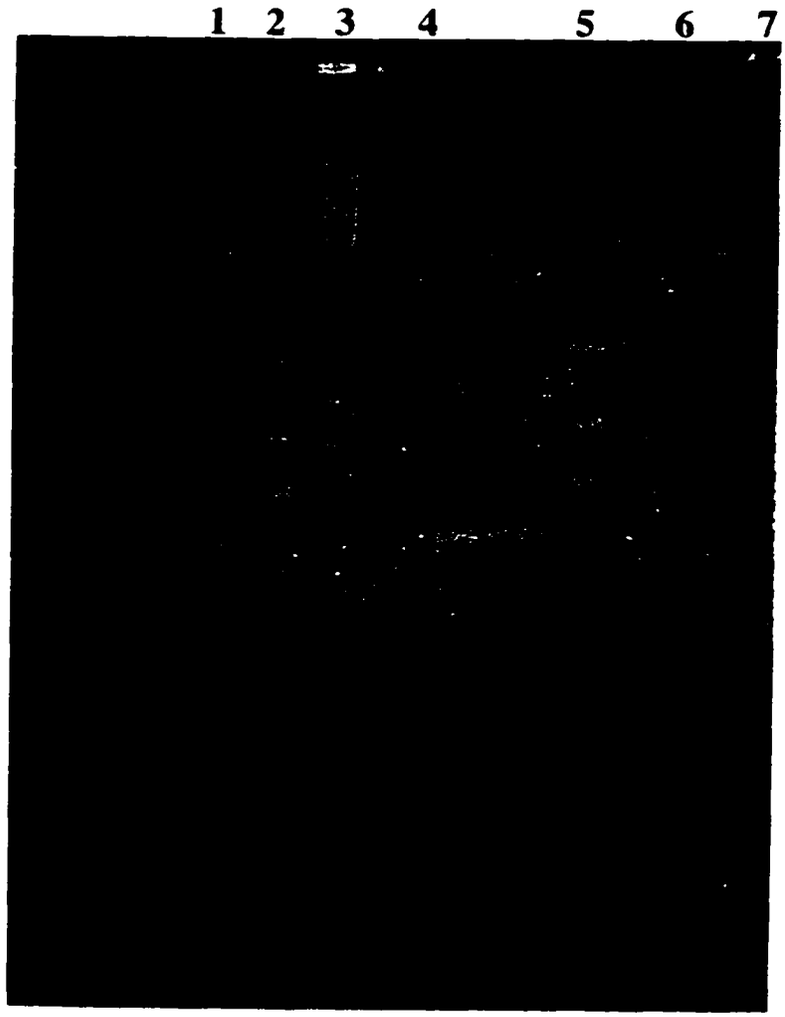
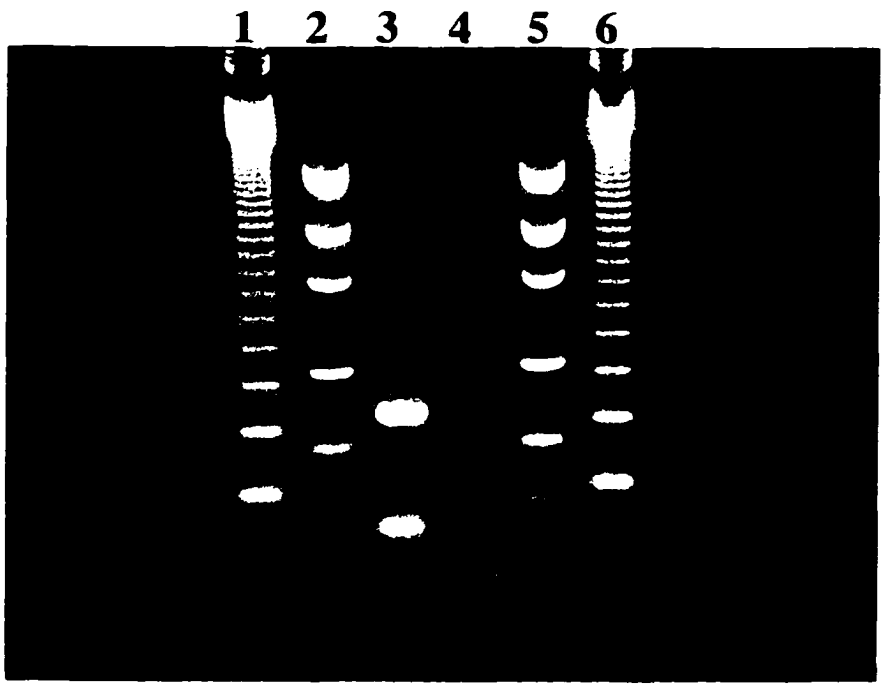


Figure 4.7 Sequence information of exon 5-6 using the reverse primer (NH2R)



Agarose Gel Electrophoresis of the cloned PCR products (JMH1-1) digested with EcoRI. Lane 1, 4 and 6: JMH1-1 restricted with EcoRI, Lanes 2 and 5: Low DNA Mass Ladder, Lanes 3 and 7: 123 base pair Marker

Figure 4.8 Restriction digest of the cloned PCR product spanning exons 5-6 of *Gambusia affinis*



Agarose gel electrophoresis of PCR products (NHF7-NJBR1) Spanning Exons 7-8. Lane 1 and 6: 123 base pair Marker, Lane 2 and 5 Low DNA Mass Ladder, Lane 3: 350 base pairs of *Gambusia affinis* DNA corresponding to Exons 7-8

Figure 4.9 PCR detection of exons 7-8 of p53 gene in *Gambusia affinis*

the combinations that had the PCR buffer {300 mM Tris-HCl, 75 mM $(NH_4)_2$ SO_4 }at pH 9.0 and 9.5 respectively with 3.5 mM $MgCl_2$. This indicates that a high concentration of Mg^{2+} was necessary for the amplification of the region corresponding to exons 7-8 (3.5 mM) as opposed to exons 5-6 (1.75 mM). PCR buffers with different pH levels seemed to facilitate amplification as was visualized as bands on the agarose gels. In addition to providing the Hot start for the reactions, Mg^{2+} levels were titrated from 1.5-3.5 mM. This added feature simplified the hot start process as both the Hotstart reaction and titration of $MgCl_2$ can be conducted at the same time. On heating the thermal cycler at 95 °C for 2 min, the wax bead melts and a vapor barrier is created. This wax layer protects the reaction mixture against evaporation losses that commonly occur when the thermal cycler is heated to high temperatures. The wax bead does not interfere with the amplification process and molecular grade $MgCl_2$ ions are released only after the wax bead melts. By withholding the essential reagents (Mg^{2+}) until after the first heating step helped minimize unwanted enzymatic activity.

With the use of the OptiHotStart™ start kit from Invitrogen (Carlsbad, CA) 10 ng of genomic DNA was sufficient for amplification in the regions corresponding to exons 7-8 as opposed to 1 μg of genomic DNA as recommended by the manufacturer. The PCR products were sequenced on the ABI PRISM™ 310 automated sequencer (Figure 4.10a) from Perkin Elmer according to the protocol described in section 3.1.1.5. The PCR product was resequenced to confirm the accuracy of the sequence readout and is included as Figure 4.10b. The sequences were aligned with rainbow trout (Figure 4.11). Remarkable homology was observed with the *Gambusia affinis*

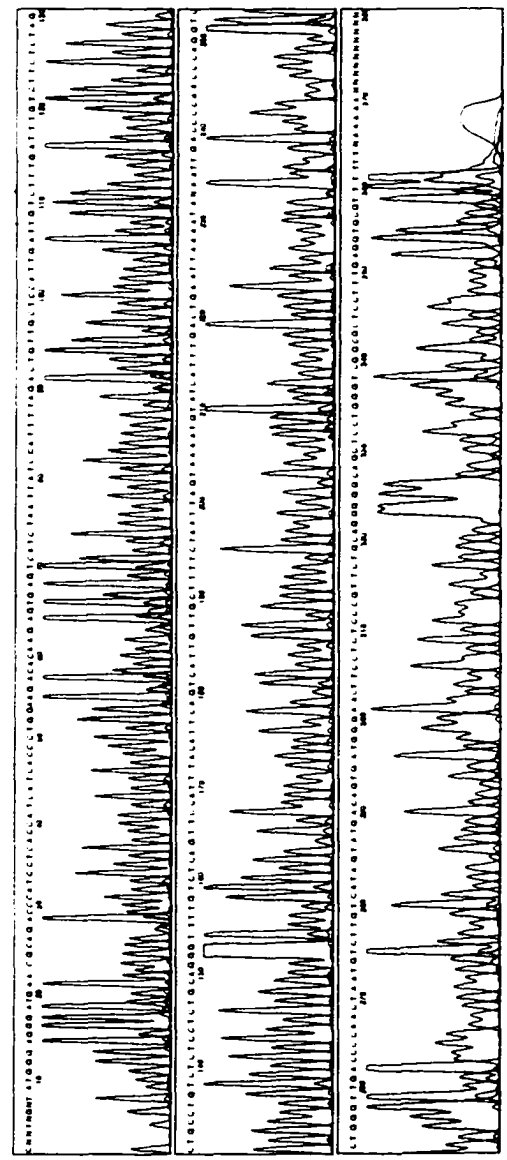


Figure 4.10a DNA sequence of *Gambusia affinis* p53 gene spanning exons 7-8

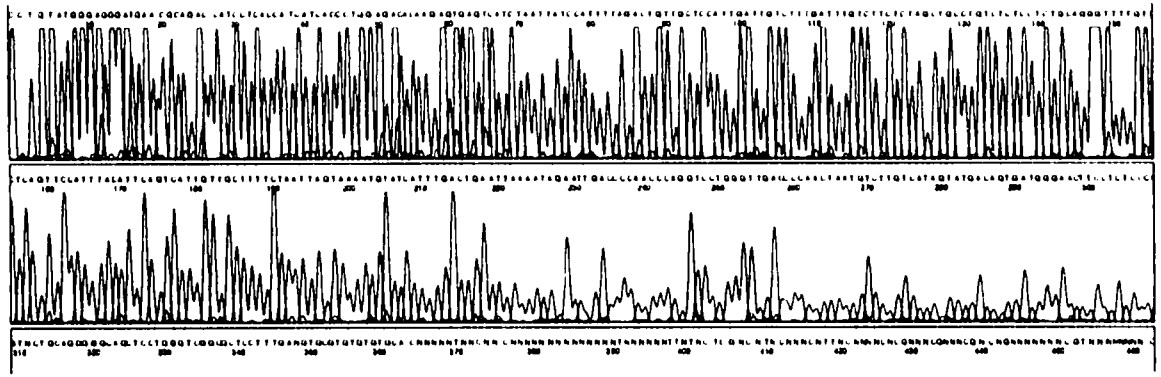


Figure 4.10b DNA sequence of *Gambusia affinis* p53 gene spanning exons 7-8 (repeat)

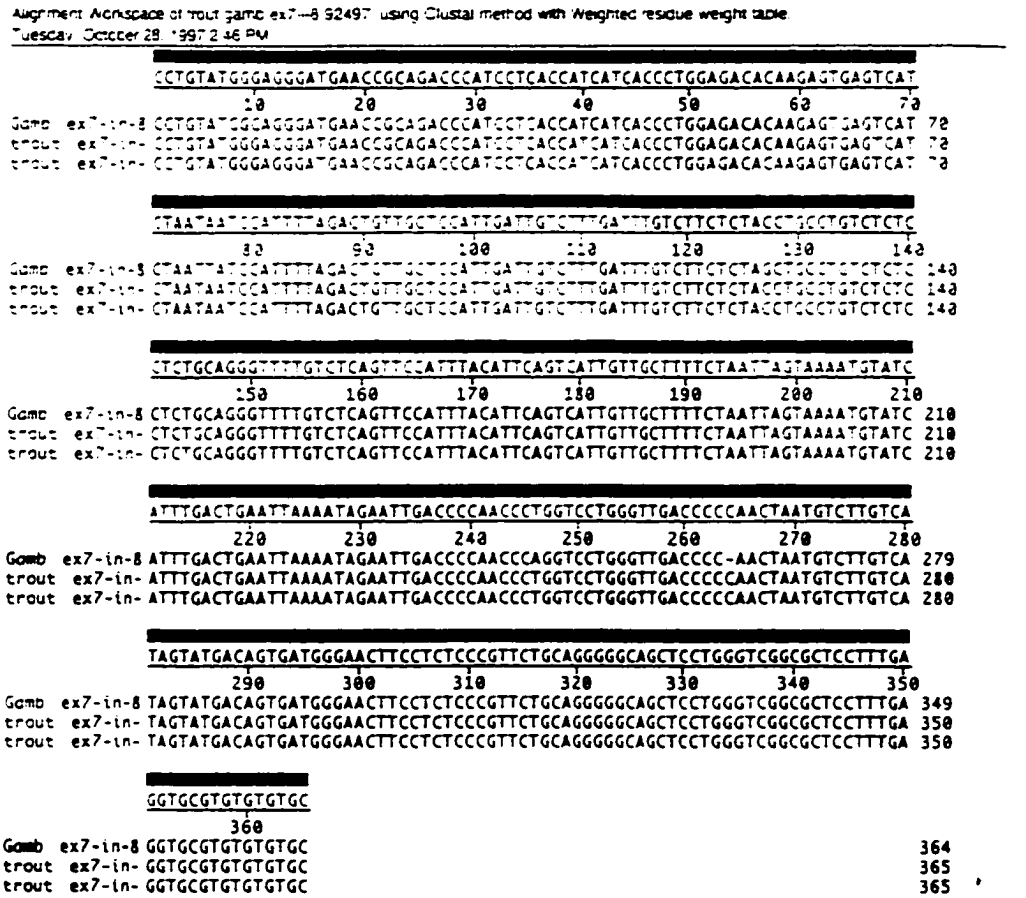


Figure 4.11 Alignment of sequences corresponding to exons 7-8 of *Gambusia affinis* and Rainbow Trout

sequences and that of the rainbow trout even for exons 7-8. The two domains characterized by exons 5-8 was identified by Greenblatt et al (1994) as the hot spots for missense mutations. The homology between the species was also evident on alignment with medaka and human p53 gene sequences (data not included).

Each of the five conserved domains in p53 gene is supposed to correspond to an exon. Specifically domains I, III, IV and V correspond to exons 2, 5, 7 and 8 of the p53 gene (Soussi et al, 1990). However, domain II is specified by exons 4 and 5. (Soussi et al., 1990). Strong evolutionary conservation was reported between medaka and other vertebrate species (Krause, 1997). In terms of amino acid homology, medaka p53 gene showed 50% homology with rainbow trout and *Xenopus laevis*, and 42% homology with human and rat. The greatest homology of medaka and human p53 cDNA was observed for domains III (87.5%) and IV (81.8%). This pattern of high homology is similar to the p53 gene sequence of exons 5-8 of the mosquito fish obtained in our laboratory.

The primary goal of this study, to obtain the wild-type sequence of exons 5-8 in *Gambusia affinis* was initiated because this part of the gene encompasses the evolutionary conserved region in all the species in which p53 gene sequence was reported. The high homology of the p53 gene for exons 5-8 in *Gambusia affinis* with human and rainbow trout p53 gene exons 5-8 is very important because, while the p53 gene itself is implicated in the majority of the human tumors, it is the exons 5-8 that actually undergo alterations in the nucleic acids (Hollstein et al., 1991, Levine et al., 1991).

The region corresponding to exons 7-8 was further sequenced using nested primers within the earlier exons (NH3 and NHBR1; Table 3.1). Data not included.

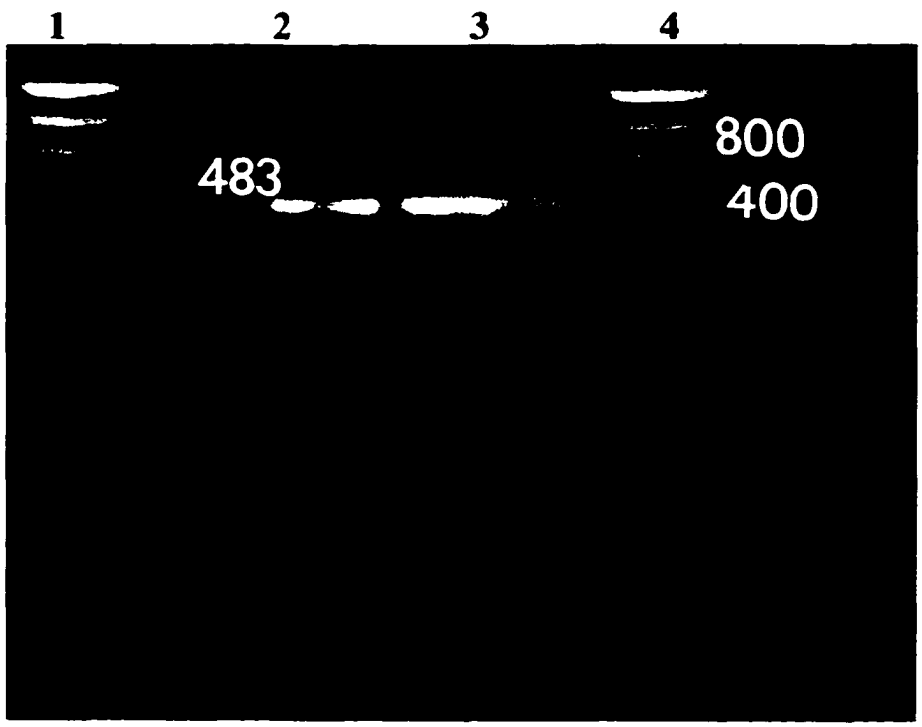
All the sequences compared very well to each other and aligned well with the trout and human p53 sequences. A few other primer combinations were selected in exons 5-6 and 7-8 in addition to NH1, NH2R, NHF7 and NHBR1. Although these primer combinations resulted in a PCR products that were estimated to be the correct size based on DNA size markers, they did not sequence properly. In some situations, this was more often the case when the same primer was used for PCR and sequencing. An excess of primer remaining after purification prior to sequencing can result in the carry-over of the primer into the sequence reaction. During cycle sequencing when the same primer or a new primer is added, the primer-template ratio is altered sufficiently to compromise the signal of the dye-labeled products. In many instances, a single PCR product was not produced and DNA extraction from agarose gels had to be performed (section 3.1.1.5). Although the protocols for DNA extraction from agarose gels were meticulously followed, and checked on agarose gels for recovery of the DNA, the quality of the sequence data was not good and difficult to interpret. One of the reasons for this could be that the ethidium bromide staining of agarose gels does not reveal the single stranded products that are present near the PCR product of interest. Also, these single-stranded products are not recorded on the gel photograph that on sequencing resulted in more than one peak at each base making it difficult to interpret the data. To resolve this problem the PCR products were purified using polyacrylamide gels in the AMBE method described in section 3.1.1.5 (Williams et al., 1995). This method of purification is simple, rapid and inexpensive. The sequence data is easy to interpret, as there are no overlapping peaks. However, in other situations, the problem was not resolved even with the help of a sequencing primer that was selected internal to the PCR

sequence. In such situations, the primer combination was abandoned or the primer was used in conjunction with another primer.

Direct sequencing of the PCR products and the cloned PCR products based on the Sanger dideoxy chain termination method (1977) are described in detail in section 3.1.1.5. The ABIPRISM™310 sequencer used has several advantages over manual sequencing and they include: use of fluroscently labeled dideoxy terminators (dye terminators) instead of radioactive isotopes, automation of the whole process that results in a simpler and rapid technique that is repeatable and hence can be tested for accuracy by repeating the process more than once. The manufacturer (Perkin Elmer, Applied Biosystems, CA) supplies Ready Reaction kits with the Termination mix that contains the enzyme (AmpliTaq DNA Polymerase, FS) and the dideoxy terminators. These dye terminators function by attaching a different fluorescent dye to each of the four dNTPs. Minute amounts of template when added to the Termination mix is subjected to undergo cycle sequencing or amplification sequencing (Grompe, 1993). Cycle sequencing requires less amounts of template, enzyme, and primers and hence is cost effective.

4.1.2 Construction of cDNA Sequence

Reverse Transcriptase in conjunction with PCR (RT-PCR) is a powerful tool to detect mRNA's. Following the RT-PCR method detailed in section 3.1.2, a cDNA of size ~ 480 base pairs was generated. Positive control with Rainbow trout also resulted in a cDNA that was similar in size to mosquito fish cDNA (Figure 4.12). However, on sequencing the cDNA of mosquito fish, the sequence did not align well with the rainbow trout cDNA. Regions that did not align well followed regions of homology. It is



Agarose gel electrophoresis of cDNA of *Gambusia affinis* (483 base pairs). Lane 1 and 4: Low DNA Mass ladder, Lane 2: 483 base pairs of RT-PCR product of Rainbow trout, and lane 4; 483 base pair RT-PCR product of *Gambusia affinis*

Figure 4.12 RT-PCR detection of p53 cDNA spanning exons 5-8 of *Gambusia affinis*

often assumed that by selecting primers from different exons, amplification of genomic targets of similar size can be prevented. However, this hypothesis ignores the presence of processed pseudogenes that can result in PCR fragments that are similar in size to that expected from cDNA (Menon, et al., 1991). The RT-PCR situation of *Gambusia affinis* is similar to the reports of Menon, et al., 1991 where they assayed RNA samples for cytoplasmic β actin mRNA using the RT-PCR method. A product similar in size to the one expected by RT-PCR was obtained. However, the sequence differed from the expected β actin sequence by 10%. A computer search confirmed the sequence to be a processed β actin pseudogene. Menon and colleagues attribute the reasons for this discrepancy in the sequences to a contamination in RT-PCR. The source contamination could be the RNA samples, the cDNA or the pseudogene. In response to the presence of pseudogene in *Gambusia affinis* and fish in general, it was the opinion of Dr. Soussi (personal communication) that none existed.

4.2 Southern Hybridization and Detection

4.2.1 Labeling Purified fragment with Digoxigenin-11dUTP

PCR products corresponding to exons 5-6 and exons 7-8 were purified and labeled according to the protocol described in sections 3.2.2. The amount of newly synthesized probe depends on the amount and purity of the template DNA. The colorimetric method was chosen to determine the optimum concentration of the DIG-control probes (Table 3.3) by comparing the color development of DIG-labeled probes with that of the DIG-control probe in a dot blot followed by immunodetection with the color substrate NBT/Xphosphate. Serial dilutions were conducted ranging from 1

ng/μl to 0.1 pg/μl. On comparing the color development of the serial dilutions of both the DIG-labeled probes with the DIG-control probe, the 10 pg/μl concentration (1:500 dilution) was found to be the minimum concentration necessary for color development in the presence of the substrate mixture. The color development at this concentration was comparable in both the DIG-labeled probes and the DIG-control DNA (Figure 4.13). This indicates that the 10 pg/μl (1:500 dilution) to be the optimum level to be used in the hybridization experiments.

- **Estimating the Yield of DIG-labeled DNA**

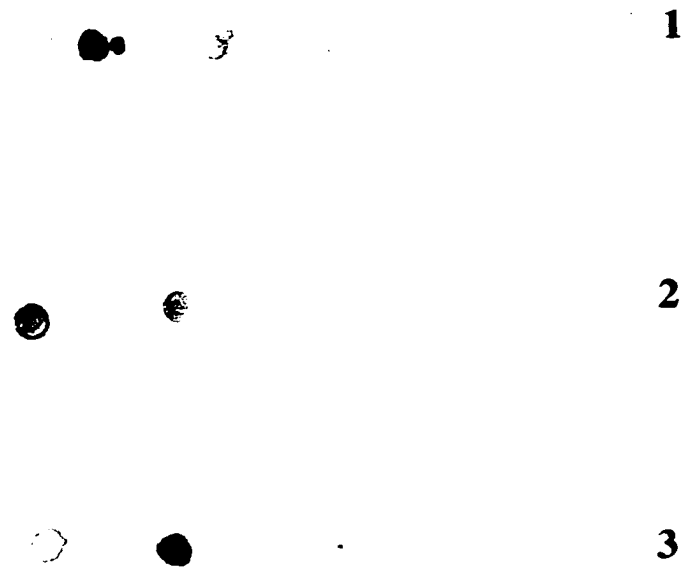
10 pg/μl * 500 (dilution factor) = 5000pg/ μl or 5 ng/ μl

The total yield of the newly labeled probe is the concentration of the DIG-labeled DNA times the volume of the probe resuspension. As the probe was resuspended in 50 μl TE/SDS buffer, the total yield is 5 ng/μl * 50 μl = 250 ng/μl

The advantages of determining the optimum concentration of the probe by the colorimetric method include minimizing the wastage of the probe and improving the quality of detection by reducing the background signal during hybridization and subsequent chemiluminiscent detection.

4.2.2 Southern Hybridization and Chemiluminiscent Detection

Southern hybridization followed by chemiluminiscent detection was performed and detailed procedures are explained in section 3.2. These procedures were attempted on a parallel scale with PCR and sequencing to confirm the presence of the p53 gene for exons 5-8 in *Gambusia affinis*. The high specificity of the DIG-labeled probes and the highly sensitive detection procedures of the Genius labeling and detection kit (section 3.2) was the reason that prompted the detection of the p53 gene in



Lane 1: Concentrations of the DIG-Control probe (1ng/ μL to 0.1 pg/μL)
Lane 2: Concentrations of the DIG-labeled probe (1ng/ μL to 0.1 pg/μL) from Exons 5-6 of *Gambusia affinis* DNA
Lane 3: Concentrations of the DIG-labeled probe (1ng/ μL to 0.1 pg/μL) from Exons 7-8 of Rainbow trout DNA

Figure 4.13 Colorimetric detection of the optimum concentration of the DIG-labeled probes

Gambusia affinis. Detection of single copy genes in total genomic DNA has been reported using this system (Feinberg and Vogelstein, 1983). In the first southern transfer experiment, genomic DNA of *Gambusia affinis* was restricted using EcoRI and BamHI separately as well as together. The PCR product of exons 5-6 from *Gambusia affinis* was DIG-labeled (section 3.2.2) and used in the hybridization experiment. On viewing the results of hybridization experiment after chemiluminiscent detection, it was found that only the fragment restricted with both EcoRI and BamHI hybridized with the PCR product corresponding to exons 5-6. This indicates that the fragment cut by both these enzymes is homologous to the fragment corresponding to exons 5-6 and that the region must be having the restriction sites for EcoRI and BamHI (Figure 4.14).

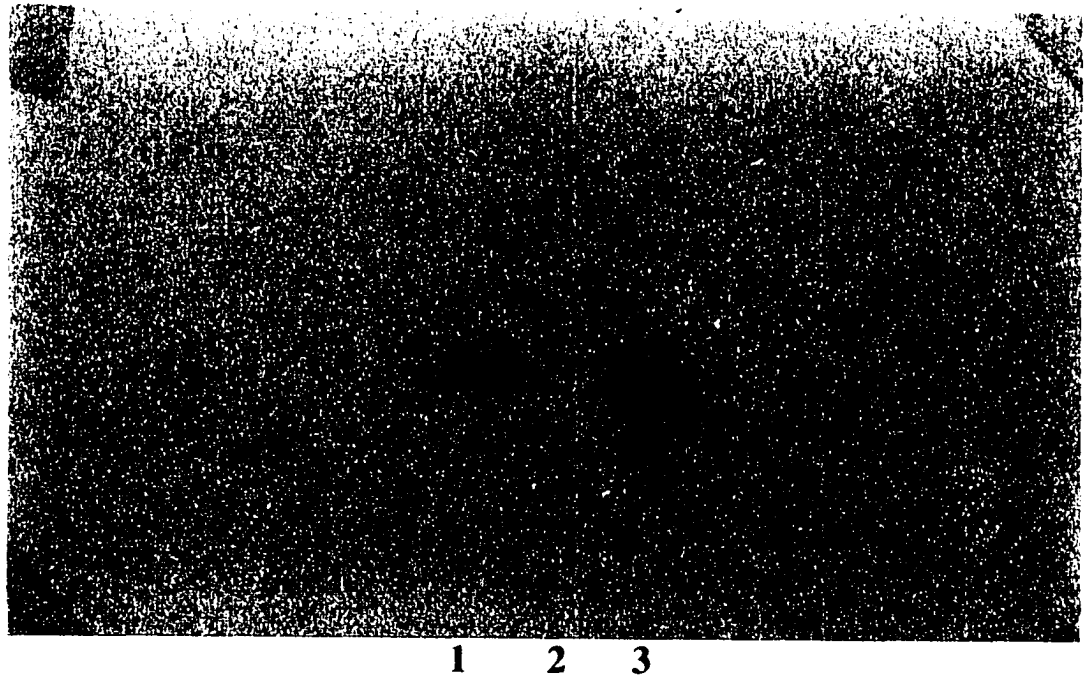
In the second southern transfer experiment, the PCR product of exons 7-8 (7F-NJBR1) from rainbow trout was DIG-labeled (section 3.2.2) and probed with the PCR product of exons 7-8 (NHF7-NJBR1) of *Gambusia affinis*. The forward primer (NHF7) in the case of *Gambusia affinis* is internal to that of rainbow trout (7FP). Chemiluminiscent detection was performed as described in section 3.2.4. Exposure to X-ray film for 30 min. resulted in visualization of a strong band indicating homology between the species (Figure 4.15). The band corresponding to *Gambusia affinis* was at a lower level when compared to that of rainbow trout indicating a smaller fragment reflective of the internal primer used. A shorter exposure (10 min.) was also sufficient for the visualization of the band that indicates that the probe was labeled efficiently and that there is sufficient homology between the two species.

To increase the stringency of the hybridization the membrane was washed at 65 °C and buffers were freshly prepared at the concentrations recommended by the

1 2 3 4 5

Lane 1: *Gambusia affinis* DNA restricted with EcoRI,
Lane 2: Genomic DNA *Gambusia affinis* restricted with BamHI, and
Lane 3: Genomic DNA of *Gambusia affinis* restricted with EcoRI and BamHI,
Lane 4: Uncut Genomic DNA of *Gambusia affinis*,
Lane 5: PCR product of *Gambusia affinis* corresponding to Exons 5-6

Figure 4.14 Detection of p53 gene (exons 5-6) in *Gambusia affinis* via Southern hybridization followed by chemiluminiscent detection



Lane1: Rainbow trout PCR product (7FP-NJBR1),
Lane 2: Genomic DNA of *Gambusia affinis*, and
Lane 3: PCR product of *Gambusia affinis* (NHF7-NJBR1).

Figure 4.15 Detection of the p53 gene (exons 7-8) in *Gambusia affinis* via Southern hybridization followed by chemiluminiscent detection

manufacturer (Boehringer-Mannheim). Prehybridization was conducted for 2.5 hr at 50° C to minimize non-specific binding of the antibody and to decrease the back-groundsignals. On viewing the X-ray film after exposure, the signal appeared sharp and bright and was visible as a single band for exons 7-8 (Figure 4.15) and was not confounded with the background signals. In case of the X-ray film corresponding to exons 5-6 the band was visualized as a relatively wide band (Figure 4.14). Using a larger electrophoresis chamber so that the bands can resolve over a longer length of the gel could modify this. However, there was no background signals to confound the re-sult. The short time (10 min.) of exposure indicates that the homology between rainbow trout and mosquito fish is high. The high labeling efficiency of the probes as indicated by the colorimetric method for the optimum concentration determination was mainly dependent on the purity and amount of DNA fragments.

The protocols for Southern hybridization and chemiluminiscent detection were standardized over a period of time by skilled technicians of the Hansen's Disease Center who were very kind in sharing the information and providing the reagents for the experiments.

Chapter 5

Conclusions

This research was conducted to test the utility of the Western mosquito fish, *Gambusia affinis*, as a sentinel fish species for genetic environmental monitoring. To achieve this goal, the specific objectives were 1) to isolate and identify the p53 gene in exons 5-8 of the *Gambusia affinis*, and 2) to confirm the presence of the p53 gene via Southern hybridization and chemiluminiscent detection, A summary of the research is presented in Section 5.1. The contributions of this research are summarized in Section 5.2. The extensions and future work possible in this research are explored in Section 5.3.

5.1 Summary

A holistic approach to better understand the processes involved in carcinogenesis includes exploring all factors and the mechanisms for cancer formation. Despite several milestones that have been attained in the process of understanding the mechanisms of cancer formation, an element of mystery remains which eludes a cure for the disease at the present time. Although germ-line mutations occur, there is overwhelming evidence of the involvement of environmental contamination as the cause of the majority of human cancers (Doll and Peto, 1981). As part of this holistic approach is the emphasis of research like this work which aims at trying to understand the mechanisms of cancer at the gene level in a model organism.

A review of literature pertinent to the present research was presented. The major areas included were: process of carcinogenesis (Harris, 1991), selection of the p53 the tumor suppressor gene as a biomarker (Hollstein et al., 1991, 94; Soussi and May, 1992; Soussi and May, 1996; Vogelstein and Kinzler, 1992), identifying the mosquito fish as a potential sentinel species in which to isolate and characterize the p53 gene (Law et al., 1994, 95 and 97), and use of PCR (Mullis and Faloona , 1987) followed by direct sequencing for obtaining the p53 gene in the mosquito fish.

The high cost of conducting experiments, issues of animal rights in the use of mammalian models and the lack of a sentinel fish in which the p53 gene has been identified and which could be studied both in the field and laboratory for environmental monitoring has sparked the interest for the search for such a fish. Many investigators have studied mosquito fish for insecticide susceptibility and resistance (Ferguson et al., 1966, Wise et al., 1986) and toxicity of various chemicals (Diamond et al., 1989, Naqvi and Hawkins, 1988, Law et al, 1994, 1996 and 1997). They have classified mosquito fish, as being sensitive to many carcinogens, easy to maintain, economical, and resistant to a wide variety of diseases. The latter group of investigators (Law et al., 1995) reported development of liver neoplasms and p53 over expression in mosquito fish exposed to the carcinogen MAM-Ac. However, the studies were limited to immunohistochemistry and DNA damage was documented in terms of adduct formation.

The results obtained from these experiments show that the region encompassing exons 5-8 of the p53 gene in *Gambusia affinis* could successfully be obtained by PCR amplification of genomic DNA followed by sequencing. Cloning of the PCR

products was also conducted prior to sequencing in some cases. The sequences from exons 5-8 of *Gambusia affinis* were aligned with trout sequences from the same regions and were found to have high homology.

In a separate set of experiments, the presence of the p53 gene in *Gambusia affinis* was confirmed *via* Southern hybridization followed by chemiluminiscent detection. A 450 base pair PCR product of exons 5-6 of *Gambusia affinis* p53 gene was generated and DIG-labeled. This was probed with genomic DNA of *Gambusia affinis* restricted with EcoRI and BamHI both individually and in combination. Also, the PCR product of exons 7-8 of rainbow trout p53 gene was DIG-labeled and probed with the PCR product of exons 7-8 from *Gambusia affinis*. The results independently confirm the high homology between the DIG-labeled probes and DNA extracted from *Gambusia affinis* confirming the presence of the p53 gene.

5.2 Contributions

A step towards further strengthening the concept of mosquito fish as a sentinel species for environmental monitoring would be the knowledge of the wild-type sequence of tumor suppressor genes such as the p53 gene.

p53, the tumor suppressor gene is highly conserved in vertebrates (man-fish). It has been identified in 23 species to date and sequenced completely only in five species (Soussi and May, 1996). Among fish, p53 gene sequence information is available in trout, medaka and mosquito fish (from this project). Therefore, obtaining the sequences corresponding to exons 5-8 of the mosquito fish (*Gambusia affinis*) represent an advance.

Alignment of the sequences of the exons 5-8 of the p53 gene in mosquito fish, trout and man indicated high homology. This region also contains the "hot spots" for mutations in man. For that reason, this region in mosquito fish could also be associated with mutations. This information is very important for focussing the search for locating base substitutions following carcinogenicity tests.

Optimization of PCR, a crucial component for the success or failure of amplification of gene sequences was accomplished in this project.

Conducting Southern hybridization and chemiluminiscent detection corroborated the presence of the p53, tumor suppressor gene, in *Gambusia affinis*.

Success of a hybridization experiment is measured in terms of absence of background variation and false positives. This is indicative of the extensive optimization of the DNA labeling and hybridization techniques. This information can provide impetus for conducting similar tests in other species to confirm the presence of the p53 gene

The various characteristics of the mosquito fish (native to U. S, small size, resistant to diseases and ability to produce tumors in a short time) are summarized in Table 5.1 in comparison with the mammalian models (rat and mouse) and fish species (trout and medaka) in which the p53 gene had already been identified. The relevance of the research undertaken in this project was compared with the results of the similar studies from literature

In summary although trout are good model for carcinogenicity testing, they require special holding rooms with adequate space as these fish grow to a size of 200 to 300g. In addition, water needs to be maintained at specific temperatures (10-16 °C),

and the large size of tissues requires extensive sectioning during histological analysis creating problems for tissue enumeration (Metcalfe, 1989).

Medaka on the other hand is a small aquarium fish like mosquito fish, however, since it is an exotic species to the U. S., it has to be restricted for use in laboratory testing and cannot be tested in the field conditions where contamination actually occurs.

Use of the mammalian models is being re-considered nowadays due to the issue of animal rights and the high cost of maintenance for conducting carcinogenicity testing.

The mosquito fish (*Gambusia affinis*) has proved itself as an effective biological agent to control mosquitoes (Dees, 1961) and hence can function in a dual role of halting the spread of diseases such as malaria and as a sentinel species for environmental contamination. The former role can be accomplished by introducing the fish in water bodies (ditches, ponds, etc) and the latter role can be achieved by periodic sampling of the fish from contaminated waters and obtaining the sequence in the exons (5-8) of the conserved regions and comparing the same with the wild-type sequence obtained in this project.

This dissertation and the previous reports of the mosquito fish in carcinogenicity testing would provide the necessary data for supplementing trout, medaka and the mammalian species as bioassay models.

Table 5.1 Relevance to research

	Mammalian Species Rat, and Mouse	Rainbow Trout	Medaka	Mosquito fish *Gambusia affinis*
P53 gene	Complete Gene	Exons 5-8 from genomic DNA and cDNA	cDNA	Exons 5-8 from genomic DNA
Method of identification	PCR and Direct Sequencing	PCR and Direct Sequencing and cDNA library screening	cDNA library screening	PCR and Direct Sequencing
Mutational Analysis	Yes	No	Yes	Yes
Sentinel Fish Species	Not Applicable	Native	Exotic	Native
Small fish	Not Applicable	No	Yes	Yes
Field and Laboratory	Both	Both	Only Laboratory	Both
Restrictions	Animal Rights	Cold Temperatures	Laboratory	None
Cost	High	Medium	High	Low

5.3 Future Research

- PCR can be used for detection of novel genes. However, for detection of p53 gene it has been used in few mammalian species and trout (only for exons 5-8). Designing of primers is an important issue in optimization of PCR. The high homology of the gene in these regions can make the designing of universal primers possible that can then help elucidate the sequence of the gene in other species. The ability of PCR to conduct amplification in a short time can help accomplish the task faster.
- Knowledge of the wild-type sequence of p53 gene in exons 5-8 would encourage other investigators to expose mosquito fish (*Gambusia affinis*) to other carcinogens to document any changes in wild-type sequence.
- Translation of the nucleotide sequence to the amino acid sequence can result in the development of specific antibodies for the p53 protein in *Gambusia affinis*. This would facilitate development of immunohistochemistry assays for mosquito fish.

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Appendix A

Preliminary Results

A.1 Mutational Analysis

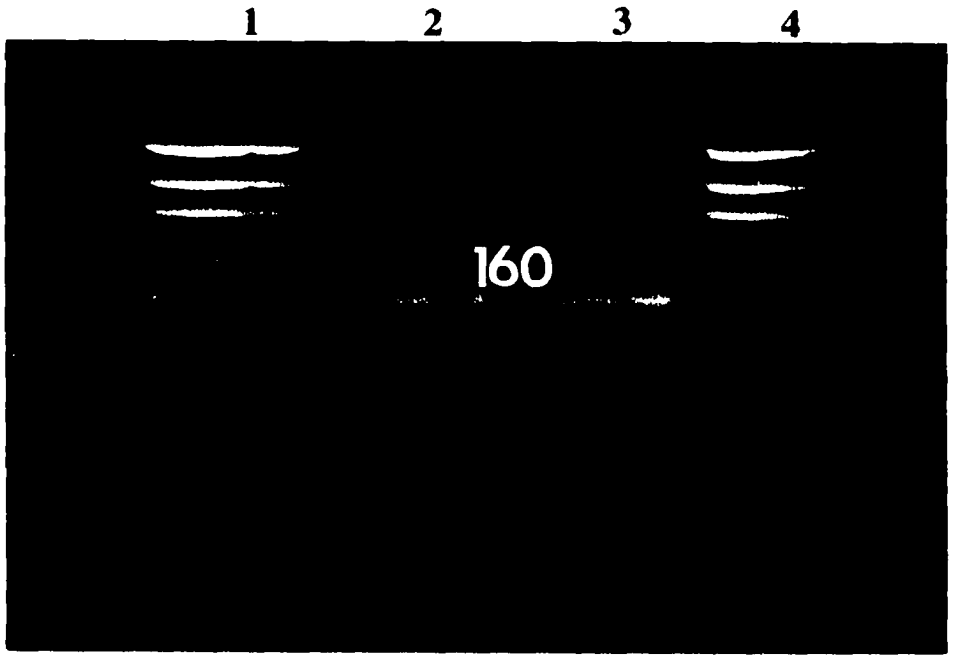
In an effort to look for mutations in tumors of *Gambusia affinis* exposed to MAM-Ac, DNA was extracted from tumors embedded in paraffin. The tumor samples used in the study were obtained from Law et al., 1994, who conducted immunohistochemistry to check for overexpression of p53 protein. PCR followed by sequencing was conducted in exons 5-6 (sections 3.1.1.2-3.1.1.5). The use of PCR to amplify DNA extracted from paraffin embedded tissues was attempted as it provides a relatively simple and sensitive technique to conduct retrospective analysis of tumor tissues (Shibata et al, 1994). As PCR analysis can be conducted on even minute amounts of DNA, it is a useful technique in such situations where the starting tissue for DNA extraction is a tumor tissue ranging in size from 5-10 μm. Also, DNA quality in terms of high-molecular weight is not a requisite for PCR and even partially degraded samples can be amplified and studied for mutational analysis. This property is aptly exemplified when one views the diverse areas in which PCR applications are seen (e.g. forensic laboratories, studies of fossils, and in parentage testing). PCR is dependent on a range of variables that need to be optimized to obtain the desired product. In laboratories that routinely perform large studies many a time the tissues are processed in formalin and embedded in paraffin with the view that the tissue can be archived for mutational analysis whenever necessary. However, this is not the case in many

situations, where mutational analysis are performed on samples that were not initially processed for retrospective analysis using molecular biology techniques. For this reason, studies of PCR analysis of templates obtained from PET have had variable success. The fixation process of a tissue is reported to be a major component affecting the quality of DNA extraction that could be used for PCR analysis. Although earlier in the chapter a statement was made on the ability of the PCR to amplify DNA from degraded tissues, incomplete extraction of salts and ethanol can act as inhibitors of the PCR. According to Greer et al., 1990, knowledge of the processing conditions that are conducive to amplification must be available to a researcher to plan studies that require using samples from earlier studies in retrospective analysis. Knowledge of the best methods of fixing tissues is very critical to investigators in fields such as molecular biology, zoology, evolutionary biology, and archeology (Greer et al., 1990). The protocol for DNA extraction from PET followed in our laboratory was obtained from protocols that used buffered neutral formalin (personal communications, Malarkey, NIEHS) for fixing tissue as opposed to Z-fix (Anatech, Ltd) that incorporates zinc formalin as used by Law et al., 1995. There was difficulty in the amplification of DNA extracted from PET and amplification was observed only with one of the sections of the tumor. The question that remains at this point was whether the zinc in the buffer acted as an inhibitor of the PCR. Reports from literature indicate fixative agents to be the primary cause of success or failure in an amplification reaction. Greer et al., 1990 summarized the pros and cons of fixatives and their success in PCR using PETs. However, the Z-fix (Anatech, Ltd), used by Law et al., 1994 for the mosquito fish samples was not mentioned in the article. The tumors from mosquito fish were fixed

in zinc formalin by Law et al., 1994 because the ionized zinc can purportedly preserve antigenic sites in the tissues by inhibiting crosslinking by formaldehyde thereby optimizing immunohistochemical studies. The length of fixation is another factor that can determine the amplification efficiency. The longer the fixation time, the less efficient the subsequent amplification (Greer et al., 1990). Also, the amount of tissue and the time between sampling was also reported to be important in determining the integrity of the sample. Dubeau and associates (1986) report that the large molecular weight DNA cannot be recovered from autolyzed and inadequately fixed tissues. In addition to these factors, there are many other factors that can act to decrease the efficiency of the amplification process. These include amplification with primers that are less efficient or degenerate. Although, this was not the case when amplification was attempted for obtaining the wild-type sequence using the same primer set, amplification was definitely not possible when the forward primer (C1) was used in conjunction with other reverse primers in exons 5 and 6 respectively. The other variables that could play a part in confounding the reaction could be the proteinase K volume in the digestion buffer. Inaccurate concentration of proteinase K in the digestion buffer or inability to inactivate proteinase K can result in the samples not being completely digested and hence not suitable for amplification. The amount of template, Mg^{2+} concentration and the annealing temperature can all affect the amplification process.

A tumor tissue is a clone of cells that have turned malignant. When DNA is extracted from tumor tissues, the odds of locating a mutation from such a tissue should be greater than from the neighboring normal tissue that would be prone only to the probability of spontaneous mutations. DNA was extracted from tumor tissues (Table

3.5), and a PCR product of 160 base pairs (Figure 4.16) was generated using C1 and GR1 primers (Table 3.1) only for the specimen number 470. The tumor was described to be a liver carcinoma with large numbers of eosinophilic cytoplasmic droplets. The PCR product was subsequently sequenced using the ABIPRISM 310™ sequencer (Figure 4.17). The sequence obtained was compared with that of the wild-type sequence corresponding to C1- GR1 of exons 5-6. No differences in the sequence corresponding to changes such as base pair mutations were observed in this region. Similar results were reported in medaka exposed to MNNG (Krause et al., 1995). Medaka fish (*Oryzias latipes*) exposed to MNNG resulted in tumors in sarcomas in gill tissue, and squamous cell papillomas. No mutations were observed in the tumor tissues although two sites of nucleotide polymorphism were found at codons 233 and 235 in domain IV. Absence of mutations in nine spontaneous hepatocelluar carcinomas (HCCs) and 34 chemically induced HCCs were reported by Goodrow et al., 1992. Groups of mice were exposed to N-nitrosodiethylamine, 4-aminobenzene and acetylaminofluorene to generate HCCs and lung cancer. However PCR followed by direct sequencing of DNA extracted from these tumors did not reveal any mutations in the p53 exons 5-8. The authors of this study attribute the reason to the fact that the p53 gene inactivation is a late event in the cell cycle and therefore present only in a limited number of tumor cells which might escape being detected by direct sequencing. In addition, the authors report that the primer selection being restricted to exons 5-8, any mutations occurring beyond this region, would not be detected. In the present mutational analysis of tumors of mosquito fish primer selection was also restricted to exons 5-8 because prior to conducting mutational studies, it was deemed prudent to obtain the wild-type



Agarose gel electrophoresis of PCR products. Lane1 and 4 Low DNA Mass Ladder, Lane 2: 160 base pairs PCR product (C1-GR1) of *Gambusia affinis* DNA from PET (specimen 470) spanning Exons 5-6, and Lane 3: 160 base pairs PCR product (C1-GR1) of *Gambusia affinis* genomic DNA

Figure A.1 PCR detection of DNA extracted from paraffin embedded tissues (PET) of *Gambusia affinis* exposed to MAM-Ac

NNNNNNNNCTCCAGGTCGTGGTGGCCCCCNCCCCCNCCCGGGGCAGTGGTGCTAGCCCTGNCNANCTATN
ANANNCTGANTGACGTGGCTGACGTGGTGAGACGCTGCCCTCACCACCGTANCACCANCGCAGAANAA
TGAAGGTACNCAACACAGNNGGAAANGCTACTCTTACCCATACTAAAACATGTTAGATTGATCTAGA
ATGTGCTTTGATGCAGTATAGAGGGTCCACACTATAAAAGGAGTTTAAACGTCATTTTATACTTACA
CTTGTTGCCTATTGAATGAGTAACGCAAATGTTTTGACTGATAGGATATCCTCAGTGTGTCGCTTAAT
ACTCNTGTTTTCTGTGCCTTA

Figure A.2 Sequence of PCR product of exons 5-6 of DNA extracted from paraffin embedded tissues

sequence. For that reason, the primary objective of our study was to obtain the wild-type sequence of the p53 gene in the mosquito fish in exons 5-8. In addition, the mosquito fish were exposed to MAM-AC only for a short period of time after which the fish were moved to grow-out chambers for tumor development. If the p53 gene inactivation is a late event as suggested by Goodrow et al., 1992, then there is a possibility that mutation detection might not be possible by this method.

Direct sequencing of PCR product was chosen over other methods such as Single strand conformational analysis (SSCP) (Orita et al.,1989) and heteroduplex analysis to detect mutations because the other methods do not define the nature of the change. Even if the other mutational analyses are followed the final step has to be direct sequencing to exactly define the location and nature of the base change. The fragment encompassing C1 and GR1 that spans 160 base pairs of DNA sequence was chosen for this study because fragments larger than 200 base pairs were reported to have difficulty in amplification (Paabo, 1989).

A.2 Preliminary Conclusions

Gene sequence was obtained for a region within exons 5-6 (160 base pairs) by conducting PCR and sequencing on DNA extracted from paraffin embedded tumors of *Gambusia affinis*. No differences were observed in the sequence on comparison with the wild-type sequence of *Gambusia affinis* p53 gene from the same region. Two reasons could be attributed for the absence of detection of mutations. The PCR-sequencing reaction is able to detect mutations if present only in more than 20% of the analyzed cells (Loda, 1994). Also the primer selection was restricted to exons 5-6 and

if any mutations occur beyond this region they would not be detected. (Goodrow et al., 1992). Use of SSCP (Orita et al., 1989) and multiplex PCR or PCR/RE/RFLP (Personal Communications, Wilson) could improve the odds of detecting mutations in tumors. Finally, the exposure protocol itself might not be sufficient for development of mutations in the p53 gene because inactivation of p53 is a late event in the cycle (Goodrow, 1992).

Mutational analysis (Retrospective analysis) by extraction of DNA from archived samples is a means of revisiting samples from previous experiments using new techniques. This procedure was attempted with *Gambusia affinis*. The information gained from this study can be extrapolated to study the carcinogen effects in other regions of the gene, and in other new genes.

Appendix B

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>Dear Soussi,
> I wanted to get your permission to use some of the figures you
>published in your Journal articles.
>Schematic representation of p53 (Oncogene (1990). 5: 945-952).
>
> My email address is neerajak@flash.net
>Thank you,
>Sincerely
>Neeraja

You have my permission as long as you cite the reference

sincerely

Prof. Thierry Soussi
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Vita

Neeraja Kondapi Erraguntla was born on January 8, 1966, in Visakhapatnam, India. She earned her Bachelor of Science degree in Agriculture and Master of Science degree in Genetics and Plant Breeding from Andhra Pradesh Agricultural University, India in the years 1987 and 1989 respectively. She joined the department of Agronomy at Louisiana State University in the year 1991 and earned a Master of Science degree in Agronomy in the year 1993. She joined the department of Veterinary Physiology, Pharmacology, and Toxicology at the School of Veterinary Medicine at Louisiana State University. She conducted research under the guidance of Dr. J. C. Means and expects to receive the degree of Doctor of Philosophy in May 1998.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Neeraja Kondapi Erraguntla

Major Field: Veterinary Medical Sciences

Title of Dissertation: Characterization of p53 Gene Sequence in Exons 5-8 of the Western Mosquito Fish, Gambusia affinis

Approved:

Major Professor and Chairman

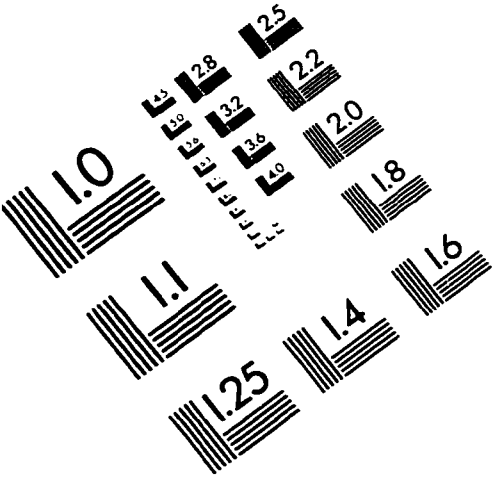
Dean of the Graduate School

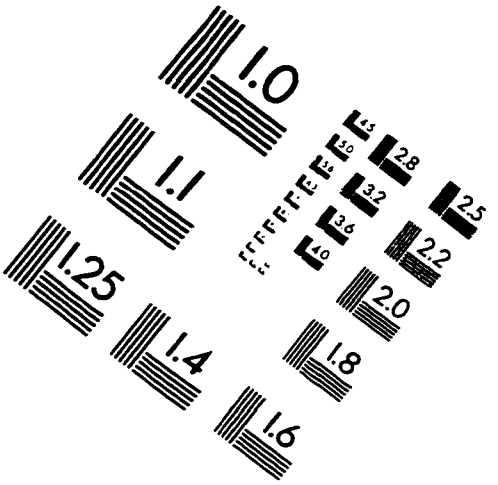
EXAMINING COMMITTEE:

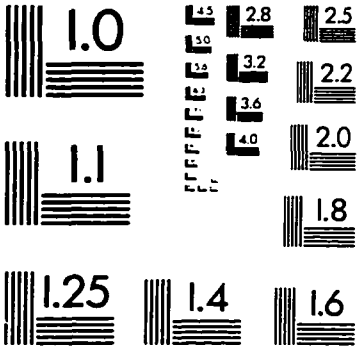
Date of Examination:

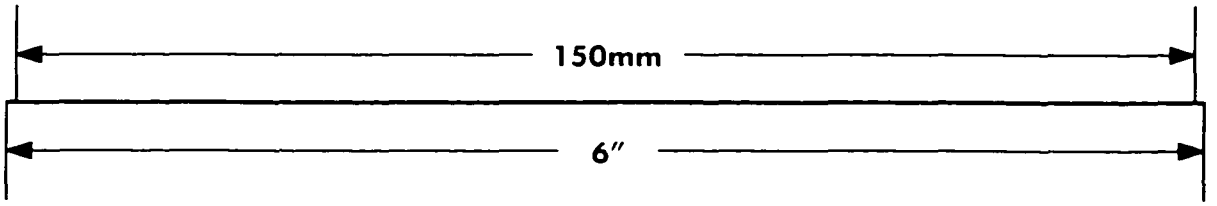
Dec. 3, 1997

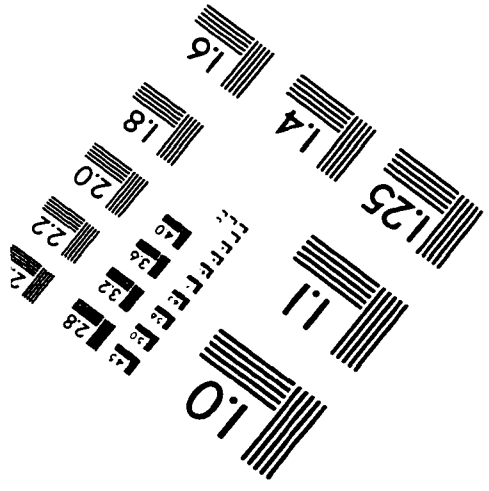
IMAGE EVALUATION
TEST TARGET (QA-3)

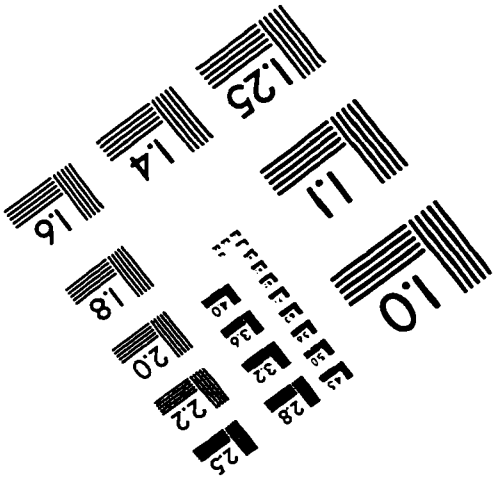












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